

nature

US bucks rules on international trade

Washington's attempts to strike a bilateral deal with Japan on trade in motor-cars are probably illegal and are certainly a threat to efforts to create a more level playing-field.

IN the now-long history of the United States, 28 June 1995 may prove to be one of those fateful dates (like 7 December 1941) after which things are never again the same. For the day just weeks ahead is that on which the United States has threatened to levy penal sanctions on Japanese motor-cars imported into the United States. Those wishing to import a luxury Toyota or Honda automobile will have to match the substantial price-tag with an equal payment to US Customs. The calculation is that hankerings after expensive Japanese cars will simply melt away; people will settle for a Cadillac (or a Mercedes) instead. This move will not seriously hurt the main part of Japanese car manufacturers' US business. But that does not mean that the penal tariffs will do no harm. On the contrary, they will irreparable damage relations between Japan and the United States. Worse, the world's system for regulating international trade will be damaged at a delicate stage in its evolution. We shall all be impoverished on that account.

The origins of the dispute about motor-cars has deep roots, but has been near the top of the bilateral agenda since the Bush administration set out to "open up" Japan's markets to outside competition. The complaints and counter-complaints are also familiar. The United States complains that Japan imports too few cars and spare parts thereof, and that Japanese manufacturers do not play the international market for car-components, commissioning US manufacturers to run off a few million door handles or ignition control systems to be assembled into Japanese cars, many of which would then be shipped back to the United States. It also grumbles at the close relationship between Japanese manufacturers and the domestic retailers of their cars; the former, the US says, require the latter to supply only authentic spare parts. (The dealers are also required to sing company songs at their manufacturers' trade conventions, but their willingness to do so does not feature on the charge-sheet.)

Japan's counter-complaints in the negotiations aborted last month are of three kinds. First, US manufacturers do not manufacture for Japan's market, witness the surprising circumstance that even European manufacturers sell a greater value of cars and car components to Japan than US manufacturers. Second, US manufacturers have not sought to increase their share of the Japanese market by manufacturing *in situ*, while the Hondas of this world have done precisely that. Finally, Japan says, complaints about the efficiency of the Japanese market for cars and car compo-

nents are beside the point so long as the international conventions on international trade say nothing about them. The last is the telling point.

From 1 January, the General Agreement on Tariffs and Trade (GATT) has been replaced by the World Trade Organization (WTO), which is endowed with all of GATT's authority and the extra rules negotiated (at the last minute, as usual) under what was called the "Uruguay" round. The old GATT rules forbid "non-tariff restraints on trade", which are usually government regulations (on safety or environmental protection, for example) that protect a national industry. But so far, nobody has thought it worthwhile to require member governments of the WTO to follow the United States in the drafting of domestic competition policy that follows in every detail US anti-trust legislation. Among other things, that would not be feasible; too many members of the WTO would say "NO!", which would be disastrous in itself.

The United States recognizes that truth, but has been seeking instead a guarantee of US sales of motor-cars and parts in Japan, much along the lines of the deal five years ago by which the Japanese government undertook to increase imports of US silicon chips to 20 per cent of the market. Japan swallowed that, but is resisting similar proposals for cars and their components. And rightly. For one thing, the Japanese government cannot tell its people what kinds of cars to buy; there is no democratic means by which that can be done. For another, a deal with the United States would inevitably cut into the business other suppliers have been able to win in Japan's market; that is why Sir Leon Brittan, with responsibility for trade at the European Commission, protested loudly at Geneva this week.

The still more serious objection to US ambitions is that they will undermine WTO at the beginning of its hazardous life. The organization was established only in January, at least partly in recognition of the need to find better ways of making a level playing-field for international traders than the spasmodic negotiations typified by the so-called Uruguay round. It is, indeed, a mechanism that could make headway in dealing with complaints that markets such as the Japanese are closed off to outsiders by hidden deals among Japanese nationals. (Most such allegations are at least exaggerated, but the issues need investigation.) By contrast, the US initiative on cars appears on the face of things to be illegal under the existing rules. If the chief economic partner of WTO rides roughshod over the agreed rules for the same of



a short-term economic gain at the expense of others, what hope can there be for a civil trading regime worldwide. And what hope, in those circumstances, can there be for a solution of persistent economic problems in rich and poor countries alike? □

Patent disputes (cont'd)

The need for changes in patent legislation becomes more clamant every day.

THE latest development (see page 348) in the long-running dispute over the patent for the polymerase chain reaction (PCR) is another indication that the world's patents legislation is due for overhaul. The patent was originally vested in the US Cetus Corporation, which sold it on to the Swiss-based company Hoffman-LaRoche for a reputed \$300 million. Key to the patent is *Taq* polymerase, a DNA polymerizing enzyme from a thermophilic bacterium. It is to Roche's credit that it seems to have recognized early on that PCR would become an invaluable laboratory technique.

Two kinds of disputes have now arisen. There are disputes about the validity of the patent (based on claims that earlier research in Russia and elsewhere invalidates the claims of Cetus) and disputes about the restrictions the patent-holders can place on the use of *Taq* polymerase. The main dispute between Roche and Promega is of the second kind; Roche alleges that Promega has been encouraging its customers buying its own *Taq* polymerase to use that for PCR investigations as well as for other purposes. These disputes will no doubt eventually be settled in the courts. So far as is known, law-suits have not yet been joined on the question of whether researchers are free to use *Taq* polymerase from any source (it can be made in the lab) for PCR without making some payment to the patent-holder. Continuing uncertainty on that score is an ambiguity in the legislation.

That is one huge uncertainty that needs to be cleared up. By now, the public purpose of the patents system is well understood. Inventors are given the exclusive right to exploit some innovation simply so that their special knowledge will be in the public domain, allowing others to prepare to exploit it (after the term of the patent) or use the special knowledge in other investigations. That has been generally understood as meaning that the patent-holder has the exclusive commercial right to use a patented process or sell a patented device, but that others are free to use the same in their own private research.

The difficulty with the PCR is that it is still predominantly a research tool. Even laboratories using PCR for genetic diagnosis on a commercial basis may be able to claim that they are in research if they contribute their data to some public database. Academic laboratories, inclined to timorousness, will necessarily be less cheeky. But under present legislation, there can be no general rule that the use of a technique in a research laboratory must *ipso facto* be free from patent restrictions. As things are, there are only the loosest definitions of what is allowed and what is not.

Legislators should also pay attention to the ambiguities arising from claims to patent rights on human genes or parts of them. Patents offices have slipped into the habit of being over-lax in their readiness to grant patents in this field. The criteria that must be satisfied if a patent is to be issued are that an invention must be original, useful and not obvious. The case for patent protection of parts of genes is that they are indeed artefacts, that they are useful (in fishing out intact genes, for example) and that (at least until three or four years ago) their potential value was not self-evident. The US patents office turned down the first application on behalf of a clutch of nucleotide sequences of "expressed sequence tags" (ESTs), but what would have been the outcome if NIH (the applicant) had sought protection for the technique? On the face of things, there is no obvious reason why it should have failed. But that would have caused more ructions than PCR has done. □

Opportunity missed

Harold Wilson had the right enthusiasms for science, technology and society, but was too impatient.

THE death last week of Lord Wilson, previously Harold Wilson and the British prime minister for two recent spells (1964–70 and 1974–76) is of more than passing interest to the research community. For was it not Wilson who startled Britain in 1963 with his speech at Scarborough promising that the re-election of a Labour government would reshape the British economy in the "white heat of a technological revolution"? What went wrong?

Wilson was a gregarious character, and many of his friends were academics. For three years before the 1964 election, he and his party set about preparing a strategy for science and technology. There were many meetings, the more formal over weekends at the not especially fashionable Hotel Bonnington. A few people, notably the physicist P. M. S. Blackett and the economist Thomas Balogh, were especially influential members of the group. They dreamt the dream of Wilson's technological revolution.

What went wrong is easily understood. Wilson was in too great a hurry. The first scheme entailed building electrical aluminium smelters that quickly proved uneconomic, while the new government poured its technical expertise into restructuring British industry with the objective of making industrial companies bigger. A key part of the argument was that only large companies could afford sufficiently ambitious research programmes. In the event, the controversial doings of the Industrial Reconstruction Corporation probably led to a shrinking of the volume of research in British industry. Curiously, much less attention was paid to the needs of British universities than was evidently required even then. And Wilson himself threw his great skill into the pressing political problems confronting his government. If he had paid more attention, and to the right things, he might have done for Britain what Mitterrand and Chevènement did for France fifteen years later. □

Brussels seeks tighter control over European funds for Russian science

London. The European Commission in Brussels appears to be on a collision course with some European Union (EU) states over the way in which it provides financial support for scientists in Russia and other states of the former Soviet Union (FSU).

Edith Cresson, the former French socialist prime minister who took over as the EU's commissioner of research at the beginning of this year, wants the ECU250 million (US\$325 million) allocated partly for this purpose within the commission's Fourth Framework research programme to be more focused on areas of research likely to benefit the economies of FSU states.

Cresson is also keen that work currently being undertaken by INTAS, the international body set up in June 1993 to administer such aid with funds from the EU and other European states, should be taken on by the commission itself. INTAS has come under strong criticism for its slowness in getting individual projects off the ground.

The commissioner's proposals will be put to EU research ministers who meet next week. At their last meeting, the ministers agreed, at Cresson's suggestion, to postpone a decision restricting their contribution to INTAS until the situation was reassessed.

But Cresson's proposals are likely to

meet fierce opposition at the ministerial meeting from those who argue that INTAS's record has been improving in recent months, and that it should now be given the chance to prove itself. Some government officials say that, whatever criticism has been made of the bureaucratic procedures followed by INTAS, these are unlikely to be reduced by transferring the administration of funds to the commission.

There is also concern that the commission's proposal to keep direct control of the allocation of funds to scientists in Russia and other FSU states may be part of a strategy to enhance its position in Brussels, a tendency which they view with suspicion.

In a statement published last week, Cresson says that the EU's support for FSU scientists should concentrate on priority topics such as environmental protection, nuclear safety, and space technology. She also says that selection criteria should be tightened,

with preference given to projects that are "closer to industrial exploitation", and candidates being required to demonstrate the potential utility of their research.

Cresson also said that it would be "more appropriate" to integrate the activities of INTAS financed by the EU within the administration of the Framework programme, and that the commission should not continue to participate in INTAS itself after the end of this year.

Jean-Christophe Filori, a spokesman for Cresson, claims that an independent audit had been unable to show that INTAS was any more efficient than the commission itself, and also revealed evidence of 'mismanagement' — such as funds being allocated to projects before all the paperwork had been completed. "We think that INTAS has been a great idea, but it is too expensive and not effective enough," Filori says.

Even supporters of INTAS acknowledge that its procedures, partly made necessary by the need to respect both the Belgium legislation under which it was set up and Russian regulations on external grants, have left many scientists frustrated at delays in getting funds to Soviet colleagues.

"This experiment has been delayed by a number of factors, which can be corrected," says Fritz Stoeckli of the University of Neuchâtel, who is Switzerland's representative on INTAS's Council of Scientists, writing in the current issue of the Soviet journal *Poisk*. "However this does not excuse the present situation, and we expect that lessons will be drawn for the future."

But, with the money at last beginning to flow, criticism is on the wane. "They are beginning to pull their fingers out," says John Osborne, a physicist at the University of Durham in England, who had been previously experiencing long delays in obtaining promised support for collaborators in Moscow.

Next week's meeting will, therefore, be vital for the future of the organization. Filori says that, even if INTAS were to close down, some of its 30 staff members would be transferred to the commission, and all existing commitments would be met.

But INTAS points out that it has almost eliminated its backlog of grants, and has recently signed an agreement with Russian authorities that should remove some previous obstacles. With Cresson already facing a rebuff over an earlier bid to reserve ECU700 million left unallocated within the Framework budget for industrial projects, the struggle over INTAS could become a test of political will between the commission and EU members.

David Dickson



Cresson: claims funds have been mismanaged.

Nature heads ISI citations survey

London. A survey of citations to papers published in three leading science journals has revealed that papers in *Nature* are referred to more frequently than those in its two main rivals in all major fields of research.

During the period 1981–1994, *Nature* had a greater citation impact — more citations per paper — than either *Science* or the *Proceedings of the National Academy of Sciences (PNAS)*, according to data published in the June issue of *Science Watch*, published by the Institute for Scientific Information.

The survey covered seven subject areas in the physical and biological sciences in a series of five-year blocks. *Nature's* impact in the biological sciences rose from just under 30 citations per paper between 1981 and 1985, to about 60 between 1991 and 1994. Biological citations from *Science* rose from 20 to 50 per paper for the same period.

In the physical sciences, the two journals were neck-and-neck for most of the 1980s. But while *Nature's* citations continued to rise — from seven per

paper in 1981–85 to about 19 by 1991–94 — *Science* has been steady at about 15 since 1988. Citations from *PNAS*, though consistent throughout, were much lower than the other two, at 20 in the biological sciences and less than 10 for the physical sciences.

Detailed breakdowns revealed immunology to be *Nature's* most-likely cited field with over 90 citations per paper. (*Science* had 70 citations.) In molecular biology, *Nature's* citations-per-paper score rose sharply through the early 1980s, but fell off in the latter part of the decade, to be surpassed by *Science*. But *Nature* re-established its pre-eminent position in the late 1980s.

In chemistry, *Nature's* impact scores substantially lagged behind those of *Science* for much of the 1980s. But its citations have risen steeply since then, and now stand at just under 30 per paper. "Carbon chemistry, and, particularly, papers on C_{60} and the other fullerenes helped lift *Nature's* impact in this field," the survey reports.

Ehsan Masood

Research reactor claims agreement on uranium fuel

Munich. A controversial research reactor planned by the Technical University of Munich in Garching seems certain to be built after a decision by the Wissenschaftsrat, Germany's science council, to recommend that the federal government pays half of the DM665 million (\$445 million) costs.

The decision has been welcomed by the university, which told the Wissenschaftsrat that it had been guaranteed 400 kilograms of highly enriched uranium (HEU) — enough to run the reactor for ten years — from the French company Cerka. The supplies have been obtained through the European Union's Euratom Supply Agency, which claims that they come from non-military sources in western Europe.

The Green party and the opposition Social Democrats, both of which have campaigned against the research reactor — known as FRMII — are concerned that the reactor will burn weapons-grade HEU. They point out that the decision to build the reactor may be in conflict with an international agreement reached in 1978, the RERTR, (Reduced Enrichment for Research and Test Reactors), that research reactors should convert to low-enriched uranium, in order to eliminate trade in bomb-grade plutonium for civilian use.

The United States has refused to supply fuel to the FRMII, leading to a search for other suppliers. Many believe that the supplies now agreed will come from HEU that has been separated at the Dounreay reprocessing plant in Scotland from unused fuel elements originally manufactured for the high-temperature reactor (THTR-300) in northwestern Germany, which was abandoned shortly after starting up in 1985.

The Wissenschaftsrat said last week it saw no scientific reason to reverse its recommendation, made in 1989, to build a new national neutron source and believed that the FRMII met the necessary criteria.

The production, transport and use of the HEU will be under the joint control of Euratom and the International Atomic Energy Agency (IAEA). The university says that there is therefore no danger that it could be used to create nuclear weapons.

It also says that it has an agreement that spent fuel will be stored at an interim storage site in Ahaus, in northern Germany, for up to 40 years. A decision on long-term storage cannot be made until Germany makes up its mind on the long-term storage of radioactive waste in general.

The final hurdles facing the FRMII are the need to obtain a licence to use nuclear material, and clearance from the local environment ministry. Both are expected by the end of the year.

Alison Abbott

Berlin university protests at bid to cut science faculties

Munich. East Berlin's Humboldt University has reacted angrily to a suggestion that it shoulder more than half of a DM47 million (\$33 million) cut being imposed by the city on its three universities as a result of its rapidly deteriorating finances.

The university, one of the principal scientific institutions in Germany before the Second World War, is also upset at a suggestion by the city's science minister, Manfred Erhardt, that it should absorb the cut by reducing its natural sciences faculties.

A meeting of the university's academic senate held last week formally rejected Erhardt's suggestion, agreeing only to cuts of DM11 million — a sum it feels would not damage individual faculties. The senate said it would take two years to decide where further cuts could be made.

But Humboldt's stand has brought it into direct conflict with the city's other two universities — the Free University, founded in the early 1950s, and the Technical University — which feel that the Humboldt has already benefitted substantially from reunification.

After the fall of the Berlin wall in 1989, the city, burdened with the costs of rebuilding its eastern sector but deprived of its generous subsidy from Bonn, soon found that it could not afford to run all three institutions. A plan aimed at reducing overlap by merging or closing faculties was approved by the parliament earlier this year after two years of bitter debate. This will save DM130 million in the combined universities' budgets over the next eight years, and reduce the number of students from 115,000 to 100,000.

Most of the reductions will be borne by the Free University, which will have to make cuts of nearly DM75 million. In contrast, the budget plans of the Humboldt University, which has already had to shed 3,500 staff during the reunification process, was left intact. But, in the light of the city's worsening financial crisis, the Berlin government has now asked the university for a further saving of DM45 million.

This time, Erhardt has decided that Humboldt University should take the lion's share of the cuts. Referring to a review carried out by the Wissenschaftsrat, Germany's science council, he has suggested the university's pharmacy faculty should be closed, and that other natural sciences fac-

ulties be reduced in size.

Konrad Seppelt, vice-president for science and research at the Free University, backs Erhardt's decision. "The Humboldt University has had enormous financial investment in medicine and the humanities [since reunification]", he says, "so natural science can never be a priority for it". By



Seat of learning: Humboldt University is appealing to its past scientific reputation to defend itself against cuts.

contrast, he argues, the huge post-war investment in his own university's science faculties must be protected.

Erhardt, who is a Christian democrat, has expressed concern that if the universities do not present him with concrete plans showing how they could make the cuts he has proposed, the parliamentary finance committee will have to delay a decision on the university finance budget until after the elections in October.

Bert Flemming, a lecturer at the Humboldt University who is also speaker on science for the SPD fraction in the Berlin parliament, agrees with Erhardt that such a delay would mean only bad news for universities, because Berlin's deepening financial crisis means that the next government, predicted to be a social democrat-green party coalition, will need to find easy ways to impose more cutbacks.

But he offers a less confrontational suggestion for reaching agreement on faculty cuts, suggesting that departments in the same discipline from different universities should agree jointly on how cuts should be shared out between them.

Two weeks ago, he says, the chemistry departments of all three Berlin universities, plus that of nearby Potsdam University, met amicably to consider the requirement to reduce the number of student places offered in the region. But Erhardt has rejected this solution, claiming that it would conflict with constitutional requirement on the way that universities are run.

Alison Abbott

US Senate votes surprise reprieve for NIH. . .

Washington. In a formidable vote of confidence in the biomedical research community, the US Senate has overwhelmingly rejected a proposal from its own budget committee to cut the future expenditure of the National Institutes of Health (NIH) by 10 per cent.

Last week, the Senate approved by 85 votes to 14 an amendment from Senator Mark Hatfield (Republican, Oregon) restoring \$7 billion of proposed cuts over the next seven years. In a week when both House and Senate passed budget resolutions cutting discretionary spending virtually across the board, the NIH was the only agency in the entire federal government to have cuts restored in either chamber.

Even Pete Domenici (Republican, New Mexico), chair of the budget committee, voted for the amendment to a resolution which he himself had put forward. According to biomedical lobbyists, who said after the vote that they were surprised at the scale of their victory, Domenici privately blamed his own over-zealous committee staff for drawing up a resolution which

assumed the 10 per cent NIH cut.

The amendment will restore NIH funding to \$11.2 billion a year — just short of this year's level of \$11.3 billion. The money will be diverted by cutting 0.6 per cent from all other discretionary programmes, apart from those of defence, health and education. Non-NIH science programmes will share the burden: the National Aeronautics and Space Administration, for example, will lose about an extra \$80 million a year as a result of the Hatfield amendment.

Harold Varmus, director of NIH, said after the vote that he was "very pleased with the support that the senators have shown". But he added that it was "important to remember that this is still early in the [1996



Hatfield: admits 'robbing Peter to pay Paul'.

fiscal year] appropriations process."

During the debate, not one senator spoke up for the NIH cut, which Hatfield branded "a prelude to disaster" for medical research. Robert Byrd (Democrat, West Virginia) did oppose the amendment, observing that it "in effect, merely rearranges the deck chairs on the *Titanic*."

Hatfield said that he had wanted to offset the cut against the entire budget, including military expenditure, but that such a proposal would have only won about 20 votes. He therefore offered a proposal that would take the money from a much smaller pot of non-military spending. "It is a matter of robbing Peter to pay Paul," he said. "Not my preference, but the only way I could find to salvage NIH."

The Senate budget resolution now has to be reconciled with the House version, which proposes cuts of \$500 million from the NIH next year. Detailed allocation of funding to NIH and other agencies will be carried out by the appropriations committees in both chambers before a budget is finally agreed in September.

Colin Macilwain

. . . but climate agency now finds itself on chopping block

Washington. The National Oceanic and Atmospheric Administration (NOAA), a key performer and user of environmental sciences in the United States, faces virtual dismemberment under proposals to disband its parent agency, the Department of Commerce, that are under discussion in Congress.

A bill published last week by Dick Chrysler (Republican, Michigan), chairman of a task force of newly-elected House Republicans which has been appointed to draw up a plan to dismantle the department, would require many of NOAA's scientific laboratories to be sold to the private sector.

But NOAA officials are sceptical that buyers would be found for the laboratories, which have played an important role in, for example, the discovery of the hole in the ozone layer and linking its expansion to the now-banned chlorofluorocarbons.

House panels are currently discussing the closure of various government departments, including Energy and Education. But Commerce is the most vulnerable, as Republican majorities in both the House and the Senate are committed to its abolition.

NOAA takes up 40 per cent of the Commerce Department's budget, and Chrysler is looking to the agency for about one-third of the savings — a total of \$2.3 billion over five years — that would be obtained by closing the department. NOAA's budget was \$2 billion last year, of which about \$600 million was spent on research.

Chrysler's bill would close NOAA's Office

of Oceanic and Atmospheric Research completely, on the grounds that its activities are "duplicative" or "better served by the private sector". It would offer NOAA's dozen environmental research laboratories — half of them based in Boulder, Colorado — for sale to the private sector through a clearing house set up to sell off unwanted parts of the Department of Commerce.

The bill would also eliminate NOAA's role in pollution research and its work on assessing the erosion of estuaries and coastlines. Other scientific functions of the agency, including its work on fisheries and weather forecasting, would be reduced in scope and transferred to the Interior Department.

Doug Hall, deputy administrator of NOAA, says the private sector will have no interest in buying laboratories whose main interest is "conducting science for the next century". He says that the agency is already planning to reduce its 14,000-strong workforce by one-sixth by 1999.

Hall points out that NOAA has already moved to save money on weather satellites by sharing data with the Department of Defense, and has made progress in collecting user fees for some of its scientific work.

The Office of Oceanic and Atmospheric Research will spend \$260 million this year on research, about \$110 million of which will be distributed to universities through grants and other partnership arrangements. NOAA officials say that less than a quarter of this programme would survive the impact

of Chrysler's proposals.

Kathy Sullivan, chief scientist at NOAA, says that a recent review of the agency's laboratory network — which included the environmental laboratories and 29 fisheries laboratories — enabled the agency to fine-tune its research priorities, but found little duplication between facilities.

She denies that the wide distribution of laboratories is ripe for rationalization. "When your research obligation deals with all the fish stocks in the United States you can't do it out of one laboratory in Kansas City," says Sullivan.

A companion bill to Chrysler's will soon be introduced into the Senate by Spencer Abraham (Republican, Michigan), and is expected to offer a similar prescription for NOAA. This is despite the fact that the chief motive for shutting the Commerce Department is its involvement in a raft of activities, such as support for advanced technology projects, to which the Republicans are ideologically opposed.

Congress has not yet shown any indication that it wants to shut down much of the science behind weather forecasting and fishery protection. The budget resolutions passed by House and Senate already assume cuts — of 11 and 5 per cent respectively — in NOAA's budget for next year. The agency has plenty of friends in Congress, and these will now try to ensure that its science base is not eroded by much more than the lower figure, whatever the future of the department to which it currently belongs.

C. M.

Promega, Roche clash over use of *Taq* in labs

San Francisco. More than 150 researchers from institutions across the United States have been dragged unwittingly into the legal battle between Hoffman-LaRoche and Promega Corporation over the rights to sell the thermostable enzyme *Taq* polymerase.

In court papers filed in US District Court in San Francisco on 15 May, Roche and its subsidiary Roche Molecular Systems Inc. of New Jersey identify the scientists as having "infringed" its patent on the enzyme by using an unauthorized *Taq*, produced by Promega, for experiments using polymerase chain reaction (PCR) techniques.

The list was drawn up by Roche at the court's direction as part of a suit which it filed two years ago against Promega, claiming that the latter company had broken an agreement not to sell its own *Taq* to scientists for use in PCR, the rights to which Roche purchased from Cetus in 1992 at a cost of \$300 million.

William Linton, president and chief execu-

utive officer of Promega, claims that listing the scientists represents an attempt by Roche to "exert control over US science and intimidate and direct huge profits from American scientists and their institutions".

But Kathy Ordonez, president of Roche Molecular Systems in Branchburg, New Jersey, said the company does not intend to involve the scientists listed in any litigation. "The issue before us is to resolve the matter with Promega," says Ordonez, describing Promega's attempts to stir up concern in the scientific community as "unethical".

Roche's list identifies scientists from institutions such as the Scripps Research Institute, the University of California, the Howard Hughes Medical Institute and the National Cancer Institute who have acknowledged in published papers that they have used the Promega enzyme in PCR.

Promega says that accusing those on the list of infringing the patents on *Taq* challenges US patent law and practice "which

permit scientists engaged in basic research to use patented products and processes without restrictions and without being subject to licensing fees".

Others say they are alarmed by the broad implications of Roche's action. "Governments, universities and private institutions that pursue knowledge for its own sake and publish freely [must now worry about] whether every tool, every operation, every reagent, is under some patent," Arthur Kornberg, Nobel laureate and professor emeritus at Stanford University, said at a press conference called by Promega in San Francisco last week.

He cited various researchers who, he claimed, have received letters from Roche accusing them of patent violation. One group from North Carolina State University complained in a letter to *Science* in 1993 that Roche would not allow them to make their own enzyme even though they would not be able to continue their work.

But Ordonez claims that Linton's charges "do not accurately reflect US patent law". She also says that various disputes with university research groups had been settled "amicably", and that it was not Roche's policy to be concerned with researchers in individual laboratories.

At the same time, Ordonez declined to comment on whether Roche feels that the use of *Taq* for PCR by basic researchers should be exempt from the requirements of US patent law. "It's extremely complex," she said, suggesting that individual researchers consult with the licensing departments of their university or research institution about the situation. "I cannot speculate on everything that Hoffman LaRoche may do in the future."

The *Taq* controversy comes at a time of heated debate over whether patents on research tools are an inhibitory influence. Linton claims that his decision to stand up to Roche is based on a concern that they may be. "Promega would not be in business today if it was not for academic institutions," he said last week. "To turn our back on that would be to turn our back on the very source of innovation to which we owe our existence."

Roche sees the current issue differently. "If Promega were not conducting its illicit activities, the problem would not exist," says Ordonez.

Meanwhile, several of the researchers named on Roche's list say they were not concerned as long as Roche did not sue academics or their institutions — or tried to affect their work. "It just seems ridiculous to me," says Dennis Burton, of the Scripps Clinic and Research Foundation in La Jolla. "As researchers we do not look through catalogues to see who's got a patent on what."

Sally Lehrman & David Dickson

Gallo to set up shop in Baltimore

Baltimore. After a year-long courtship involving as many as eight suitors, AIDS researcher Robert Gallo has decided to end his 30-year association with the US National Cancer Institute (NCI) to head a new Institute of Human Virology at the University of Maryland in Baltimore. He will be joined by William Blattner, a fellow NCI researcher and epidemiologist, and Robert Redfield, a clinician at the Walter Reed Army Institute of Research.

The new institute is expected to open early in the autumn, and will take a multidisciplinary approach to the study of chronic viral diseases, with a major emphasis on HIV-1 and AIDS research. In addition to serving as director of the institute, Gallo will head its basic sciences division, while Redfield will lead the clinical division, and Blattner head the division of epidemiology and prevention.

The institute will be housed in the 200,000-square-foot Maryland Biotechnology Center, and will eventually have 300 employees. A private biotechnology company linked to the institute will also be set up, but neither Gallo, Redfield, nor Blattner will be officers in the company.

In addition to funds from the University of Maryland, the institute will receive \$12 million during its first three years from the state of Maryland and the city of Baltimore.

The financial package was not the largest offered among three other institutes that were wooing the trio — namely the Medical College of Pennsylvania in Philadelphia, the Virginia Commonwealth

University in Richmond, and the University of South Carolina in Charleston.

But the researchers say they opted for Maryland because of its leadership and infrastructure, access to an increasing



Gallo: keen to build links with clinicians.

HIV-infected population, and the opportunity to combine basic research, epidemiological and intervention studies, and clinical service under one roof.

Gallo says he expects the link to the biotechnology company to enable the institute to move findings rapidly from basic research to the clinic. "I want to control what research is going on and move it toward practical applications," he says.

In addition to HIV, researchers at the institute will study leukaemia viruses, neural virology, herpes viruses, chronic hepatitis viruses, and perhaps eventually papillomaviruses.

Blattner says he welcomes the opportunity to bring together basic research and public health. "We're very committed to a clinical program that will serve a community where HIV is greatly expressed," he says. "We hope to develop a seamless interface between patient care and clinical research."

Nancy Touchette

Top aide to face charges in French HIV blood scandal

Paris. France's HIV-contaminated blood scandal returned to the front pages last week with the decision to charge Louis Schweitzer, head of the private office of Laurent Fabius, who was prime minister when the affair occurred in 1985, with 'complicity in poisoning'.

The decision to press charges against Schweitzer — grand-nephew of the French missionary Albert Schweitzer and now head of the automobile group Renault — was announced on 19 May. Three days later similar charges were brought against Patrick Baudry, former adviser to Georgina Dufoix, who was minister of social affairs in 1985.

Both moves follow the earlier decision by French legal authorities to pursue former ministers and their staff for involvement in the alleged decision to delay the screening of donated blood for HIV in 1985.

But the decision to take legal action based on allegations of 'poisoning' continues to shock many, including some who lodged the initial complaints. More than one third of the latter have decided not to pursue such charges against Michel Garetta, the former head of the National Blood Transfusion Centre, and Jean-Pierre Allain, formerly head of research at the centre, both of whom have already been convicted for 'deception over the quality of a product'.

"It does not shock me at all that the legal authorities are trying to clarify what happened," says Axel Kahn, of the Institute of Molecular Biology at the Cochin Hospital in Paris. "But the charge of 'poisoning' totally lacks credibility."

Many believe that under French law, poisoning must include both direct 'administration' of lethal substances, and evidence of a deliberate attempt on the life of a victim.

From March 1985, the political authorities were warned of the probable contamination of blood products prepared to pooled blood donations. At the same time, the American company Abbott was waiting for authorization to distribute a screening test on the French market, while the French company Pasteur-Diagnostic was said to be putting the finishing touches to its own test.

Schweitzer and Baudry are being held partly responsible for an alleged delay in introducing the screening of blood products. Schweitzer is being blamed for protecting the interests of the company Pasteur Diagnostics, and Baudry, as a representative of his ministry, is being blamed for refusing to accept that the costs of screening should be covered by social security.

It was only on 19 June 1985 that Fabius made screening obligatory. The French test was registered on 21 June, and the Abbott test on 24 July.

Catherine Tastemain

Academy backs science in Endangered Species Act

Washington. For the second time in less than a month, a report from the US National Academy of Sciences has landed squarely in the middle of a political controversy. Early last month, an academy document on wetlands was published in the middle of a House spat over their definition. This time, the academy has stepped in with a report on endangered species during a Congressional battle over reauthorizing the Endangered Species Act (ESA).

The academy report, released last week after a two-and-a-half-year study, endorses the basic scientific underpinnings of the 22-year-old law that protects rare plants and animals in danger of extinction — despite claims by critics in Congress that the law is based on faulty science.

"I can't identify a major scientific flaw" in the act, said Michael Clegg, professor of genetics at the University of California, Riverside, who chaired the study, during a public briefing on the report. The committee did not deny that the act could be improved; but, to the chagrin of property rights advocates in Congress who would like the ESA scaled back (see *Nature*, 374, 9; 1995), its suggested fixes would probably mean even more protection for wildlife.

In order to put the listing of threatened plants and animals on a more objective footing, the panel recommends the identification of what it calls "evolutionary units," or EU. Each would be defined as "a segment of biological diversity that shares a common evolutionary lineage and contains the potential for a unique evolutionary future."

Such a scheme would be similar to that already used by the National Marine Fisheries Service — one of two US agencies charged with setting guidelines for the protection of endangered species — to distinguish among marine taxa.

But the panel reaffirms the Endangered Species Act's basic premise: that protection of subspecies and populations is scientifically justified. This has been a bone of contention in debates over the spotted owl, one subspecies of which is plentiful in Mexico, and another of which is endangered in the timber forests of the American northwest. Some critics of the ESA have argued that unless the whole species is endangered, it should not be given protection.

The report also asserts that habitat is essential to a species' survival, and thus deserves protection under the act. "The link

between species survival and habitat is indisputable," says Clegg. The US Supreme Court is expected to decide this summer whether destroying habitat can be legally considered to be harmful to a species.

The report throws cold water on the idea that captive breeding and the reintroduction of endangered species are viable alternatives to preserving native habitat. The committee accepts that captive breeding may be successful in some cases. But it recommends that it be "avoided when possible", and used only as a last resort to protect a species from extinction.

Although none of this is welcome news for critics of the ESA, the study has so far been spared the angry attacks that greeted

an academy wetlands report released earlier last month (see *Nature*, 375, 171; 1995). But property rights advocates in Congress have still tried to put their own spin on the new report.

The day after its release, for example, Richard Pombo (Republican, California) told a hearing of the House endangered species task force, which he chairs, that while it was important to consider the views of scientists, "it is our job to ensure that science is not used as an excuse or a basis for trampling on the constitutional or legal rights of any individual".

But Newt Gingrich (Republican, Georgia), the speaker of the House, who stepped into the hearing for a brief statement, was less combative, suggesting that the two sides of the ESA debate might reach a compromise. Gingrich applauded the academy report's emphasis on preserving biodiversity rather than individual species, and spoke of his own "deep concern for the biological diversity of the planet".

Perhaps it is because they hold most of the cards that conservative Republicans can afford to be gracious. Having already imposed a moratorium on the listing of new species, and proposed deep cuts in the funding of agencies such as the Fish and Wildlife Service that implement the ESA, conservative Republicans also head the House and Senate committees that are responsible for rewriting the act later this year.

Pombo describes the coming debate over reauthorization as "one of the most controversial issues we will take up as a Congress". Gingrich is calling for a revised act that is "economically rational" and "biologically correct", and safeguards private property. But even he admits that balancing such interests will not be easy. **Tony Reichhardt**

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REASONS

Caught by the act: the bald eagle is no longer endangered.

Smithsonian

Europe holds fire on spy satellite plan . . .

Paris. Europe's joint security organization, the ten-nation Western European Union (WEU), has agreed to turn its experimental satellite ground station in Torrejón, near Madrid in Spain, into a permanent facility.

But it has postponed any decision on how to proceed with related plans to develop a European military satellite system — a decision which will depend on how Europe eventually responds to offers of cooperation from the United States.

At present, the main activity of the Torrejón centre is the processing of satellite images from SPOT and other commercial suppliers, to verify arms agreements, and to monitor both security crises (for example in Bosnia) and environmental changes.

WEU is currently considering building its own satellites for telecommunications, high-resolution Earth observation, and eavesdropping. But its member states continue to balk at the costs of such a programme, estimated at \$840 million a year for 25 years.

Lanfranco Emiliani, director of Earth observation and environmental programmes at the European Space Agency (ESA), has suggested that one way to cut the costs of European military programmes would be to exploit synergy with ESA programmes.

Another option would be for WEU to obtain data from Helios-1, a military observation satellite built by France, Italy and Spain which will be launched this year, as well as other military satellites planned by European nations.

WEU has, indeed, already signed a memorandum of understanding with France to obtain some classified images from Helios-1. But officials from the Helios-1 programme are reported to be very reluctant to share data with countries which have not contributed to the costs of building the satellite.

Bhupendra Jasani, of the Department of War Studies at King's College in London, argues that WEU will wait to find out what data it will be able to obtain from Helios-1 before taking a decision on whether to build its own system.

Negotiations are further complicated by the high stakes involved in developing military satellite systems. At the political level, the major space powers — such as the United States, Russia and France — seem determined to maintain their technological lead, and to prevent the proliferation of high-resolution (1 metre) images, just as nuclear powers are keen to prevent non-nuclear states from building nuclear weapons.

This appears partly to explain the relative enthusiasm of the United States, France and the United Kingdom to cooperate on an international military telecommunications satellite programme, Immilsat, which would also take advantage of the fact that all three countries need to replace their existing military satellites around 2005.

But these countries are also determined both to protect their domestic satellite industries and to boost their international competitiveness. Germany, for example, is expected to invest in the French-led Helios-2 programme; but it has repeatedly postponed a firm commitment in what is widely being interpreted as a bid to negotiate better industrial returns from the project.

The political and industrial stakes are perhaps best illustrated by the current stance of the United States. In March, Gil Klinger, the US Defense Department's acting deputy undersecretary for space acquisition and technology, offered to cooperate with Europe on an international military satellite system, and promised that the United States would not impose undue control over any jointly developed systems.

In particular, Klinger said that President Bill Clinton supports transatlantic cooperation on a Space-Based Infrared System, already scheduled for launch in 2002 as a replacement for the US Defense Support Program's missile-detecting satellite system.

But Klinger's offer is being viewed with scepticism by those in Europe who point out that the United States faces a dilemma. If it prohibits its aerospace companies from selling high-resolution satellites to other countries, it will lose business; but such countries risk eventually finding alternative suppliers, which would result in the proliferation of high-resolution images.

The United States now seems to have decided that if it cannot prevent commercial operators putting classified images on the market, it would prefer [if such images] were being sold by US companies that the Pentagon can control during a security crisis. "The US political manoeuvring is clear," argues Stephane Chenard from the Paris-based space consultancy, Euroconsult.

Indeed, some Europeans believe that a Europe-only satellite system, known as Eumilsatcom, would be preferable, as it would both defend the local industries and make Europe less dependent on the United States for satellite data.

Barry Blaydes, the director of the Torrejón centre, says the question of whether WEU builds its own satellite system will "take time to resolve." He points out, however, that the centre can, meanwhile, continue to operate with procured images, although adding that the acquisition of high-resolution Helios data will be "an important step" in its future.

Declan Butler

. . . as CIA shows off its early efforts

Washington. Early satellite photographs of China and the Soviet Union taken by satellites launched under the once-secret Corona programme have been released as part of an effort by the US Central Intelligence Agency to explain its mission to the public.

The images include that of the Chinese nuclear test site at Lop Nur (far right) taken on 20 October 1964, four days after China conducted its first nuclear test. Another (above), taken above Moscow on 28 May 1970 with a resolution of about 6 feet, shows the queue for Lenin's tomb.

The Corona programme conducted its first successful mission in August 1960, three months after a Soviet missile had brought down the U-2 spy plane on which the United States had previously been dependent. But the first mission alone obtained more data than all of the U-2's flights had done, and for the next decade, the CIA was deluged with information.

Unveiling some of it at a symposium at George Washington University, John Deutch, professor of chemistry at the Massachusetts Institute of Technology and newly-appointed director of the CIA, praised the links between scientists, industry and government that enabled Corona to succeed.



"This team had enormous technical obstacles to overcome and no time for lengthy preliminary studies," he said.

The release of the Corona data has been long sought by vice-president Al Gore, who believes that it will be of great value to environmental scientists.

HUGO and gene patents

SIR — A recent News story (*Nature* 374, 751; 1995) attributed to the Human Genome Organisation (HUGO) the view that “patent protection of human genes is essential”. This is incorrect. The original sentence from which the quoted phrase was derived reads: “In particular, we want intellectual property rights to be allocated fairly, in a manner that appropriately weighs the contributions of different parties to the total research effort, and creates necessary incentives for the ongoing development of products without interfering unduly with scientific research.” This is fundamentally different from stating that patenting of gene sequences is essential. In context, the HUGO document on which your article is based stresses that if patenting is considered, the criterion should be functional utility rather than mere sequence by itself.

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□ How, within the present framework of patent law, would HUGO suggest “the contributions of different parties to the total research effort” should be accounted for? — Editor, *Nature*.

Virology institute changes direction

SIR — You reported (*Nature* 374, 299; 1995) the dismissal of Professor David Bishop from his post as director of the Natural Environment Research Council (NERC) Institute of Virology and Environmental Microbiology, Oxford, and published a comment from Bishop (*Nature* 374, 590; 1995).

I am writing on behalf of myself and nine colleagues*. We have been advised by the NERC that the action taken does not imply any criticism of Bishop's scientific standing. We wish to record our sympathy with Bishop and to note his scientific achievements. In the 11 years under his directorship, the institute has achieved a strong reputation, especially in virus insecticides, molecular virology and environmental microbiology. In the most recent report of the institute (1992–94) the NERC representative commented: “I find it particularly impressive that, across the whole spectrum of the Institute's activities, basic research of the highest quality is leading to potential applications of the science.” The NERC Institute of Virology and Environmental Microbiology, which is a separate institution from the University of Oxford, attracts more than 55 per

cent of its budget from contract research funded from the European Union, industry and government agencies other than NERC.

The matter raises two major issues. First, there is the question of the morality and legality of the abrupt dismissal. We understand that this is the subject of current proceedings. Second, there is the wider issue of the right way to evaluate science and to develop change. The mechanism of informed and transparent peer review is accepted by scientists as a fair way to evaluate research and to provide instruments for change, where necessary. Good science takes many years to build up but can be destroyed all too quickly. We hope that the NERC will find a way to fulfil its obligation to government but also to promote research which is at the forefront of knowledge in virology and that will ultimately benefit mankind by the improvement in understanding of basic science and the ability to exploit this knowledge.

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Neurogenetic determinism

SIR — The nature–nurture debate has engulfed neuroscience at least since Darwin's day. Rose's Commentary¹ embodies some arguments that are overstated, offering little by way of evidence to support them.

Thus he points to setbacks in attempts to identify gene markers for neuropsychiatric disorders, asserting that “at best, the hunt for genes for these conditions may be able to identify anomalous cases in which the genetic defect is to mimic a more widespread phenotypic condition”. The basis for this assertion is unclear. The early setbacks in genetic studies of schizophrenia, depression, manic depression and alcoholism appear to have stemmed largely from methodological pitfalls, not necessarily from the absence or rarity of susceptibility genes². There are now several instructive examples. The initial genetic findings in dis-

orders such as Alzheimer's disease, diabetes mellitus and breast cancer, which resemble psychiatric disorders by way of phenotypic and genetic complexity, were not always compelling or easily replicated. With further study, there is now incontrovertible evidence of single-gene subforms for these disorders.

Rose proceeds to claim that pharmacological response rather than the clinical syndrome has been made the basis of diagnosis in psychiatry. But this claim is groundless: although response to treatment is sometimes used to ‘dissect’ clinical syndromes into treatment-responsive and treatment-resistant (a common and useful practice in all medicine), the principal basis for a psychiatric nosology remains behavioural phenomenology³.

Rose refers to the “almost universal conviction among biological psychiatrists that schizophrenia is a genetic disorder” and their apparent oblivion to alternative explanations. He also claims that “it is well known that with a little ingenuity any phenotypic distribution can be explained genetically, granted appropriate assumptions about partial penetrance and incomplete dominance”, implying that geneticists manipulate data to ‘prove’ a case. But the “conviction” that schizophrenia is a genetic disorder is not universal. In fact, psychiatrists with a biological bent are all too aware that only a fraction of schizophrenic patients show high familial loading for the disorder, so that non-genetic aetiologies are at play in a substantial number of cases⁴. As for the allegedly manufactured ‘match’ between phenotypic distributions and genetic explanations, countless attempts to fit genetic models to phenotypic distributions of schizophrenia have failed to resolve the mode of inheritance⁵.

The study of brain and behaviour poses major challenges for neurogenetics. Complex phenotypes and unclear transmission patterns complicate the search for genetic underpinnings and demand full appreciation of the forces at play. The implications for society mandate caution in the interpretation of results. To ensure scientific excellence and to stave off abuse, science deserves its watchdogs. But they too should be held to high standards lest an ‘anti-science’ climate takes hold.

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

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The meaning of "person"

SIR — John Godfrey (*Nature* 373, 100; 1995) seems to admit the possibility of an "infusion of the soul". But in the next sentence, he implicitly equates the human person with the gradually evolving body. The possibility of the human person being an incarnated soul is silently dismissed. It is only the body and not the person or individuality that is seen to come "into being by continuous progression during ontogeny". If a pre-existing soul (in the Platonic sense) makes up the core of human individuality, and gradually takes possession of the evolving body, that individuality cannot be said to come into being in a continuous way, although its (new) body does.

Moreover, Godfrey states without any kind of proof that the gradual character of human ontogeny implies a gradual growth of the rights of the individual. Even from a purely materialistic viewpoint, there is no logically compelling reason why this implication should hold. Does the gradual rising of water level logically imply gradualness of the consequent inundation? Of course not: the same lump of reality can have both gradual and discontinuous aspects. Moreover, the argument is not only flawed but also antihuman. Adult members of our species are endowed with several biological characteristics such as a fully erect stance, tool fabrication and language. None of these capacities is already present in the newborn human; they evolve gradually after birth. Does it follow that newborn humans have a diminished right to live? Of course not. The simple truth is that biological systems as such never have rights, just as colours as such never have weight.

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SIR — Godfrey states that the "fundamental biological and ethical issue" (*sic*) raised by Pope John Paul II in his book *Crossing the Threshold of Hope* is "the origin of an individual person during life before birth". He furthermore states that "there is no moment when human life starts" and, further on, that "it is understandable that ethical thinking should have been founded on obsolete biology".

In his book, Pope John Paul II does not explicitly state what he regards as the fundamental biological and ethical issues in human reproductive biology, but he did write: "the concept of 'person' is not only a marvelous theory; it is at the center of the human ethos"; and, in another paragraph, "in this field more than in any other, collaboration among pastors, biologists and physicians is indispensable". Godfrey combines these two passages into a single quotation although the

two statements by the Pope were made in different contexts.

The programme for development as a human being with personhood is set in motion at the beginning of the fertilization process. The events that may potentially follow the union of end-cell gametes might not have occurred had the initial event in the fertilization process not been successful.

It is difficult to understand how Godfrey defines person. He seems to say that a person is someone who has characteristics conferred by the complete expression of her/his entire genetic endowment. If that were the situation, it seems unlikely that any person would survive long enough to attain reproductive age and that would settle the issue once and forever.

To justify his view of why there can be no such thing as person during embryogenesis, Godfrey recounts the complexities of the fertilization process, which may take several days for completion.

These events include (with a few of my own added) the activation of the ovum, chromosomal events, maternal influences, expression of paternal genes, the possibilities of twinning and entry of genetically foreign cells, organogenesis including neurogenesis, birth and the acquisition of language. This sequence of events is indeed, according to Godfrey, a "seamless change". But this is irrelevant to an understanding of what the Pope said.

Hopefully, the concept of person as expressed by Pope John Paul II will remain at the centre of the human ethos. We must not be distracted by obscurantism and the many straw-men placed here and there to guard the windows of opportunity for human embryo manipulation. Let us hope that ethical thinking may survive in the milieu of contemporary biology.

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SIR — Godfrey offers a convincing example of the incapacity of biology to represent a reference point for ethical issues. As a biologist, Godfrey states that "there is no moment when human life starts", . . . "life is continuous from one generation to the next", . . . "the picture of the nascent human which emerges from modern embryology is one of seamless change." Biology is clearly not able to establish the frontiers of life. (It is not uncommon that an expert in a field finds serious difficulties in defining his science.)

So we have to look elsewhere for more solid foundations of ethics. Empirical knowledge and good sense (*Epikeia*) give us sufficient arguments for significant statements. They teach us that when a man and woman couple, there is a high probability that the woman becomes pregnant. Nine months later a baby will be born, who will grow to be an adult. Something new surely happened after (or during) the coupling and originated something/somebody that did not exist before. That act is necessary for a new human being to be born. From then on, all that concerns the result of coupling has to do with the life of a potential person. (Of course, at this stage, he is not a person, nor is a newly born baby a person.) Therefore, and notwithstanding he is not a person in a technical sense, he has some rights, the first of which is to life. The conclusion of Godfrey from the state of his art ("the rights of, and our duty towards, the unborn must grow gradually") is hazy and ambiguous, and may lead to arbitrary behaviour.

The Commentary offers also a good example of the troubles into which a scientist falls when he tries to derive ethical issues or philosophical conclusions from the results of his researches. A scientist, lacking the sense of complexity of reality, often tends to extrapolate his results, which are valid for a given limited field, to all of reality, without considering the other sciences. It is already difficult to study irreversible transformations in dynamical systems, as from melt to solid: how much more the emergence of a new human life. A human being, even unborn, is not just an assemblage of living cells, but something other too, and other disciplines (philosophy, sociology and so on) have to be involved when studying human behaviour and discussing ethical issues.

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DNA evidence

SIR — In 1987, Andrew Deen was convicted of rape largely as a result of DNA evidence.

On 21 December 1993, the Court of Appeal in London ordered a retrial after hearing evidence from the defence (see *Nature* 367, 101–2; 1994). The implications of the retrial were widely reported and discussed in *Nature* (D. J. Balding & P. Donnelly 368, 285–286; 1994) and by the press. However, at the Liverpool Crown Court on 26 April 1995, the DNA evidence was accepted without question by the defence and Deen pleaded guilty.

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Dioxins released from chemical accidents

Andrew A. Meharg and Daniel Osborn

Discrepancies in environmental budgets of dioxin-like compounds may be explained by emissions from accidents involving chlorinated organic chemicals. This source may have important implications for regulation inventories.

FEW chemicals cause as much concern and controversy as the toxic and environmentally persistent dioxin-like compounds, a group that includes the dibenzo-dioxins and dibenzo-furans (generically known as the dioxins) and coplanar polychlorinated biphenyls (PCBs)¹. Dioxins have never been made for commercial purposes (other than as environmental standards) but they arise as trace pollutants in the manufacture, use and disposal of chlorinated organic compounds. The amount of dioxins in the environment increased markedly following the expansion of the chlorine-based sector of the chemical industry in the 1940s². From the 1950s to the late 1970s, PCBs were made in large quantities and widely used as commercial transformer and capacitor fluids, but their production is now banned in many parts of the world because of alarms about environmental contamination².

Many scientists believe that these now ubiquitous chemicals have an adverse effect on our health and environment, whereas many industrialists, with some academic support, claim that the threat is greatly exaggerated¹. The carcinogenic effect of dioxin-like compounds is hotly disputed. Laboratory studies indicate that they may cause liver cancer in rats. But epidemiological studies of humans exposed to high concentrations fail to reveal increased rates of cancer¹.

There is now more worry about the non-cancer effects of dioxin-like compounds, particularly dioxins in which chlorine atoms are substituted at positions 2, 3, 7 and 8 and the 11 coplanar PCBs that, although not substituted at these positions, have at least four chlorine atoms in positions mimicking the structure of the 2,3,7,8-dioxins (see figure over). These 2,3,7,8-dioxins and 2,3,7,8-dioxin-like PCBs are more toxic than other dioxin-like compounds. They bind to a cellular protein known as the Ah receptor, which has a vital role in the function of the immune system³. Binding triggers a series of events that includes the disruption of endocrine, reproductive and immune systems, impairment of fetal development and decreased glucose tolerance¹. Some of these effects are thought to occur at concentrations only ten times body averages¹. Human and animal populations exposed to concentrations tenfold higher than background

levels may be at risk from dioxin toxicity, particularly people exposed occupationally or accidentally or who eat large amounts of fish or beef, the main dioxin source for humans¹.

Given what we know about the toxicity of dioxin-like compounds, as well as the growing concern about their other effects (including evidence of intergenerational impacts) and the continuing controversy over cancer, the US Environmental Protection Agency (EPA) recently undertook a major review of the human and environmental effects of these chemicals.

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Clean-up after the Seveso accident in 1976.

The review attracted widespread comment. Published in a six-volume 2,000-page draft document⁴, it concludes that the risk of cancer is between 1 in 1,000 and 1 in 10,000 for high-exposure groups. Non-cancer effects are also considered to be important. It must be stressed, however, that other national and intergovernmental organizations have used different approaches in assessing the risk posed to humans and the environment by dioxin-like compounds⁴. These assessments predict that the risks from current environmental burdens are lower than suggested by the EPA review.

A wide range of chlorinated organic compounds are fundamental to our lives, including plastics, pesticides, antiseptics, lubricating fluids and bleach⁵. Altering their production and use to reduce emissions of dioxin-like compounds has had — and will continue to have — far-reach-

ing social, economic and environmental implications.

For these and other reasons, one of the priorities of the EPA review was to identify the main emissions of dioxin-like compounds into the environment. In previous reviews in the United Kingdom⁵ and Sweden⁶, the 'known' sources of dioxins (manufacture of chlorinated organic compounds, chlorinated waste incineration, wood and coal burning, metal recycling, and wood-pulp and paper bleaching) account for only about 10 per cent of depositions. The EPA review draws similar conclusions for the United States.

But these estimates are based on relatively few observations and the underlying science requires strengthening. In particular, none of the studies considers the release of dioxin-like compounds from chemical accidents. The omission of this 'accident' term in the inventories adds considerably to their uncertainty.

Large amounts of dioxins may be produced in accidents involving the manufacture and storage of chlorinated organic compounds. The manufacture of the common antiseptic trichlorophenol (TCP) has been plagued by accidents producing the most toxic of all the dioxin-like compounds, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)⁷. Indeed, dioxins were brought to the attention of the world by the notorious explosion at a chemical works in Seveso, Italy, in 1976: 0.26 kg of TCDD was found in soil surrounding the factory⁸ and up to 3 kg may have been released in total⁹. This disaster (and others) led the European Community to formulate the Seveso Directive⁸ aimed at minimizing the risks of chemical accidents. The legislation focuses on preventing catastrophic events but does not consider smaller, more frequent accidents that could also cause widespread environmental contamination.

Organochlorine pesticides such as the phenoxy herbicides contain dioxins as contaminants, and although their use is now restricted, they remain a source of emissions^{5,10,11}. Another important and so far unconsidered source is the burning of these chemicals. Combustion of pentachlorophenol (PCP) under controlled experimental conditions produces dioxins in yields of up to 45 per cent¹⁰. PCP is still widely used to treat wood. Fallout

from the smoke plume of a fire at a wood-treatment plant involving 4.5 tonnes of PCP resulted in dioxin contamination of foraging animals in the area¹¹.

Dioxins are also produced when chlorinated plastics are burnt¹²⁻¹⁴. Given the flammability of polyvinyl chloride (PVC) and its bulk use in a variety of commercial products (cable insulation, construction materials, food packaging and so on), large-scale fires at PVC storage and recycling installations are not uncommon¹²⁻¹⁵. Funcke and colleagues¹⁴ investigated 200 fires involving chlorinated plastics, and found increased concentrations of dioxins at 90 per cent of

extent of PCB accidents, listing 3,600 accidental releases of roughly the same annual amount of PCBs^{16,17}. (For 1990, however, TRI records the release of only 150 tonnes of PCBs¹⁸.)

Of the 209 PCB congeners, the 11 with 2,3,7,8-dioxin-like activity are thought to make up the bulk of the 2,3,7,8-dioxin-like compounds entering the environment, as their concentrations are much higher than those of the similarly substituted dioxins. Because accidents are responsible for the release of a considerable tonnage of PCBs each year, they are probably the largest single environmental source of dioxin-like compounds.

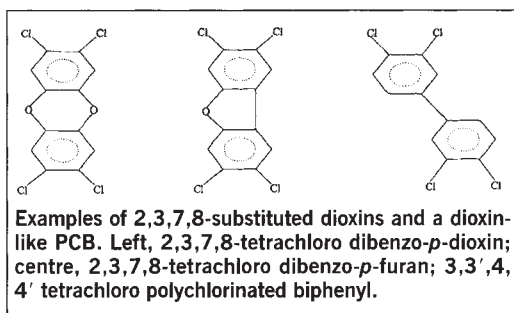
PCBs can be released through the rupture of transformers, capacitors and storage vessels during transport; in accidents at transformer and capacitor production and recycling plants; and in fires involving transformers, capacitors and PCB storage installations¹⁹. Combustion of PCB under experimental conditions yields 0.03–2.00 per cent dibenzofurans by weight, so pyrolysis of 1

technology or favouring processes with lower dioxin outputs. Accidents are more difficult to manage, particularly given the range of possible source materials and the diversity of manufacturing, storage and disposal installations. More effort must be made to prevent accidents, especially if dioxin-like compounds need to be controlled as tightly as the EPA report implies. This will involve analysis of the life cycles of chlorinated compounds to determine at which stage, if any, dioxin-like compounds can be produced. Having identified these stages (such as the distribution and recycling of PVC), efforts can be concentrated on accident prevention.

Inevitably, such reassessments of the potential for the generation of pollution during the life cycle of manufactured materials will require specialists from many different disciplines to work together to balance society's needs, the economic means of meeting them and the environmental consequences of doing so. This interdisciplinary approach is essential if we are to continue to benefit from the products of the chemical industry while protecting the environment from the harmful effects of dioxin-like compounds and other environmentally persistent chemicals. □

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the sites. On combustion of a range of PVC formulations, the total weight of released dioxins with four or more chlorine atoms varied from 1 mg (soft PVC) to 22 mg (rigid PVC) per kg of burnt material (the latter representing roughly 10–15 per cent of the original weight of the plastics)^{12,13}. A large-scale plastics fire typically involves 500–1,000 tonnes of PVC¹⁵. Using the data for rigid PVC in refs 12 and 13, it has been estimated that such a fire could produce 1.1–2.2 kg of dioxins, which amounts to 3.75–7.50 per cent of the annual input into the UK environment from all identified emissions (about 29 kg)⁵.

There is clearly a need for accurate measurement of emissions from accidents involving chlorinated organic chemicals. The task would be aided by determining atmospheric dioxin emissions for a range of incidents combined with an inventory of large accidents.

Detailed inventories for PCB accidents already exist. Two EPA databases, the Toxic Release Inventory (TRI) and the Emergency Response Notification System (ERNS), hold comprehensive information about chemical accidents in the United States. They show that large amounts of PCBs are released from accidents. TRI lists data for both accidental releases and licensed releases during manufacture. Because the manufacture of PCBs was banned in the United States several years ago, all PCB releases subsequently listed originated from accidents: for 1987 and 1988, 22 per cent of all entries are listed as releases of PCBs, corresponding to an annual total of 1,700 tonnes¹⁶. The ERNS database for 1988–1992 confirms the

tonne of PCB could alone produce 2 kg of dibenzo-furan.

Commercial PCB formulations are also a source of dioxins. One kilogram of used PCB cooling fluid contains about 10 mg of tri-hepta-chlorinated furans²⁰. The concentration of these furans in unused cooling fluids is less, ranging from 0.2 to 5.4 mg per kg for several commercial PCB mixtures²⁰. Assuming that 1,700 tonnes of PCBs are released annually into the US environment from accidents, the annual input of tri-hepta-chlorinated furans from PCB accidents is about 17 kg. This alone represents roughly 4 per cent of the maximum amount (400 kg) of dioxins estimated to enter the US environment each year from atmospheric deposition²¹. On top of this, the large amounts of dibenzo-dioxins and dibenzo-furans produced during combustion of PCBs must also be considered. In a recently published study of top-predators in an area contaminated by a PCB fire²², PCBs and dibenzo-furans were found in concentrations 2–13 times those of background.

To reduce emission of dioxin-like compounds, sources must first be identified. Although published emission inventories account for only 10 per cent of recorded depositions^{5,6}, these estimates are based on relatively few measurements. More detailed studies would help to focus strategies for further reducing dioxin emissions. The important input from accidents should now also be considered when devising these strategies.

Reductions in dioxin-like emissions have so far been achieved by concentrating clean-up efforts on identified sources mainly by tightening controls on existing

Particle physics in slivers of mica

A heroic investigation at Berkeley has shown that mica of good quality can be used to search for evidence of one possible component of the missing mass required to close the Universe; none has been found so far.

ONCE upon a time (in 1986), when the search for magnetic monopoles was fashionable, P. B. Price at the University of California, Berkeley, had the grand idea of looking for evidence of the existence of monopoles by hunting through specimens of mica. For magnetic monopoles, whose existence is required by certain unifications of particle theories, should leave tracks a few micrometres long in minerals of all kinds. Mica is an ideal material in which to look for tracks of this kind because of the ease and precision with crystals of it can be cleaved. For a time, it seemed as if it would be possible to uncover a kind of fossil record of magnetic monopoles.

Even though there are still many people who speak fondly of grand unified theories (or GUTs), the hunt for free magnetic monopoles has fallen out of fashion. (The hunt for free quarks, which some even suggested might be found by taking vacuum cleaners to surfaces to which charged particles might adhere, has been abandoned for better reason — the recognition that quarks cannot, in the present condition of the Universe, exist in isolation from other quarks.)

But the potential usefulness of mica as a permanent record of past fluxes of particles of matter has not sunk without trace. The same P. B. Price has now described the use of mica to provide a limit — not a particularly stringent one — for the past flux of particles of what are called weakly interacting massive particles, or WIMPs, that may be one component of the “missing mass” that the cosmologists need to make the Universe gravitationally closed. It is central to what follows that WIMPs are massive objects, at least as massive as neutrons; on one view, they rejoice in the name “neutralino”; neutrinos that happen to have mass would not qualify.

Perhaps the most striking feature of Price's latest account of his work (D. P. Snowden-Ifft, E. S. Freeman and P. B. Price, *Phys. Rev. Lett.* **74**, 4133-4136; 1995) is its implicit demonstration of how techniques have improved in just under a decade. In 1986, looking for micrometre tracks in sheet of mica meant pushing optical microscopy to its limits, and not then succeeding very well. Now it seems to be much easier; simply etch the mica with hydrofluoric acid and use atomic force microscopy to measure the depth of etch pits in the surface. With careful

calibration, it should be possible to tell which pits are what.

At this stage, it is probably fair to say that the group has gone little further than to define the magnitude of the background problem that must be taken into account by particle physicists who turn to mica to fill in time while waiting for the next particle accelerator to be commissioned. But that turns out to be intriguing. Although WIMPs carry no electric charge, their presence in minerals can in principle be detected by the recoil of nuclei with which they collide, and which in general will be ionized. These should leave tracks of lattice defects behind them in well ordered crystals, but stopping distances will usually be quite short.

What other generators of micrometre tracks in mica could there be? Atomic force microscopy has come into its own with what seems to be an unambiguous definition of the tracks left in mica by the decay of individual uranium and thorium atoms incorporated as impurities. Each emission of an α -particle creates a recoil nucleus, and there are eight α -decays in the uranium series before ^{238}U becomes lead, but the directions of successive nuclei are not correlated with each other. The result is that each radioactive atom generates a zig-zag of connected tracks so that the result after etching is quite a deep pit, reaching below the surface for 10 nm or more.

How in practice is it possible to distinguish between those messy and unwanted events and the simpler expulsion of a nucleus from an atom by a WIMP? That is now neatly dealt with. The first step is to cleave a crystal of mica, etch the two fresh surfaces and measure the depth of the pits in them both by atomic force microscopy. The pattern of the pits on the two surfaces can be used to put the mica sheets in proper register with each other. The general expectation is that the sum of the depths of opposing tracks in each of the two surfaces will be greater for α -recoil than for recoil of atomic nuclei caused by the collision of WIMPs.

Tracks that appear not to cross the interface or which fail to yield etch pits 2 nm deep in each of them are excluded from the measurements. And they are evidently rather time consuming. The team says with some pride that it has so far measured more than 80,000 μm^2 of surface, which is a small fraction (8 per cent)

of a square millimetre.

That is the neat part of the investigation. Then comes the messy bit. To begin with, the mica (the group uses muscovite mica) must be chosen with care. Freedom from radioactive impurities is evidently a plus, so too is age (to integrate the recoils caused by WIMPs over as long as possible). Something of the geological history of the mica is also necessary, for annealing at a few hundred degrees Celsius is enough to get rid of many tracks. The calculations are that the search for WIMPs has been carried out with a specimen of muscovite mica roughly 500 million years old (measured by *in situ* fission tracks) and that it has never been exposed to temperatures greater than 200 Celsius.

The next step is to estimate the expected background of tracks caused by α -recoil, which can be done with a knowledge of the radioactive contamination and, of course, a Monte Carlo programme. The same goes for the spontaneous fission of uranium and thorium. But what is to be said of the spectrum of pits expected from the collision of WIMPs with nuclei in the mica? The way round that problem is to expose the mica to a fast neutron flux in a reactor, letting neutrons stand for WIMPs, and then to calculate the distribution of recoiling nuclei from the known composition of the mica. Even at 200 degrees Celsius, annealing is fast enough for roughly one in three putative WIMP tracks to have disappeared in 500 million years.

How does it all turn out? Nobody would yet expect the result to be compelling, but one interesting feature of the use of mica is that the material contains roughly 5 per cent of ^{39}K , the nuclear spin of which is likely to enhance the cross section of collisions with incoming WIMPs. Even so, the authors acknowledge that their result is an upper limit on the existence of WIMPs of particular mass that is an order of magnitude less stringent than previously obtained by other methods.

But optimism is not dimmed. Price and his colleagues say that their limits can be made more stringent; “we will simply analyze more mica”. Careful selection of the mineral, from deep mines drilled into uranium-free rock, will help. So will the selection of a specimen of mica from the centre of a large block. Everybody, of course, will wish the enterprise good luck.

John Maddox

Vortex lattice melts like ice

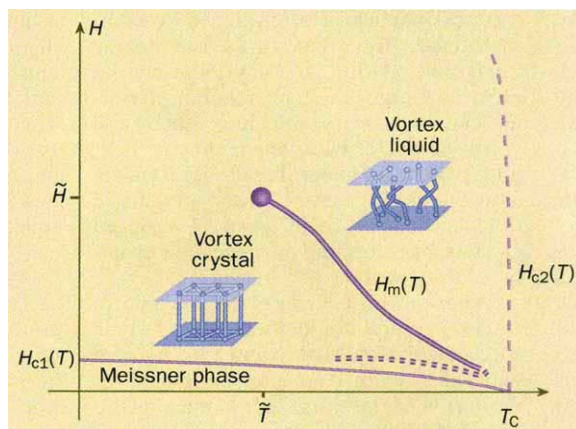
David R. Nelson

ON page 373 of this issue, a paper by Zeldov *et al.*¹ provides unequivocal evidence that the lattice of magnetic vortex filaments in a high-temperature superconducting compound melts via a first-order phase transition. This striking experiment demonstrates remarkable and unconventional behaviour of type II superconductors in a magnetic field. The results are also exciting because they confirm that the vortex liquid expands on freezing, similar to the transformation of water into ice. Such behaviour is rare in nature and shows that matter composed of flexible directed lines behaves differently from assemblies of point-like atoms or molecules.

The response of superconductors to an external magnetic field H is almost as striking as the property of zero resistance which gives them their name. Below the superconducting transition temperature T_c , small magnetic fields are expelled. The behaviour in higher fields of type II superconductors (an especially important class which includes the high-temperature cuprate compounds) is particularly interesting. As predicted by A. Abrikosov in the 1950s (see for example ref. 2), the magnetic field enters in the form of quantized lines of vorticity above a lower critical field $H_{c1}(T)$. These lines repel each other, and were assumed to crystallize into a regular lattice. As the field increases further, the vortex filaments become denser, until all trace of superconductivity is finally lost above an upper critical field $H_{c2}(T)$. Continuous, second-order phase transitions were predicted at H_{c1} and H_{c2} . Applications in the regime $H_{c1} < H < H_{c2}$, such as magnetically levitated trains and portable magnetic resonance imagers, require that the vortex lines be pinned in place by imperfections in the underlying crystal.

Early theoretical work neglected effects of thermal fluctuations on the vortex lines. In the new high- T_c materials, a combination of elevated critical temperatures, short superconducting coherence lengths and high anisotropy causes vortex crystals to melt into an entangled liquid of filaments well below the zero-field T_c (ref. 2). Melting of the fragile vortex crystals above a line $H_m(T)$ makes pinning the filaments for high-field applications much more difficult, and raises fascinating intellectual issues about the physics of tangled interacting lines. As pointed out by Zeldov *et al.*¹, the field $H_m(T)$ above which melting occurs can be three orders of magnitude smaller than $H_{c2}(T)$.

A first-order melting transition of the Abrikosov vortex lattice due to thermal fluctuations was predicted in 1985 by Brezin *et al.*³. The first experimental confirmation appeared in the compound $\text{YBa}_2\text{Cu}_3\text{O}_7$ (YBCO), which is closely related to the $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ (BSCCO) studied by Zeldov *et al.* When vortices in clean twin-free samples were finally examined^{4,5}, hysteresis loops in the resistivity as a function of field and temperature appeared, providing strong evidence



Schematic phase diagram of clean high-temperature superconductors. The triangular crystal of vortex lines melts via a first-order transition into an entangled vortex liquid along $H_m(T)$, which terminates at a critical point $(\tilde{T}, \tilde{H})$.

of a first-order transition. This remarkable hysteresis has now been confirmed in many laboratories. Nevertheless, scepticism remained⁶, in part because the resistivity is a dynamical quantity sensitive to pinning by the residual disorder present even in clean samples.

The experiment by Zeldov *et al.* puts such doubts to rest, at least for BSCCO at low fields ($H < 380$ Oe). The key advance is an exquisitely sensitive probe based on the Hall effect, which is used to measure a sharp discontinuous change in a thermodynamic quantity, the local vortex density. The experiments are at least an order of magnitude more sensitive than earlier work. Zeldov *et al.* measure a local magnetic field B (proportional to the vortex density) which jumps by about 0.5 gauss over a temperature interval of less than 3 mK at a melting temperature of about 80 K. A similar jump is observed when the transition line is crossed by varying the external magnetic field.

In the phase diagram revealed by the experiments (see figure), the most striking feature is that the melting line $H_m(T)$ terminates in a critical point. Above about 380 gauss, the discontinuity in vortex density vanishes. The abrupt hysteretic changes in the resistivity of YBCO com-

pounds also disappear above an apparent critical point⁷ and it is possible that both critical points have a similar origin. Critical points along the melting curve of most forms of matter are ruled out, because the ordered crystalline phase cannot transform smoothly and continuously to the disordered liquid.

A critical point *can* arise in vortex matter because vortex lines are always subject to residual pinning disorder. As a result, the range of translational correlations in the Abrikosov flux lattice is not really infinite, but limited instead to a maximum size determined by the disorder strength. In low fields, there is a first-order transition when the translational correlation length jumps discontinuously from somewhere around the vortex spacing in the liquid to a much larger value in the solid. The size of this jump decreases with increasing fields as the range of crystalline order in the solid shrinks. Weak disorder may wash out the phase transition entirely beyond a 'decoupling field'², which is the same order of magnitude as the field at which these critical points appear. Above the decoupling field, thermal fluctuations cause vortex lines to fragment into decoupled sheets of point vortices and become much more sensitive to residual point pinning.

The exact behaviour for fields above the critical point is still hotly debated. The simplest possibility is no transition at all, rather as a liquid becomes indistinguishable from a gas at a critical point. A more speculative proposal is that residual point-like disorder leads to a high field line of continuous thermodynamic phase transitions into a 'vortex glass' phase⁷; but detailed experiments on YBCO seem to argue against this^{5,8}. More work is needed to decide which of these interpretations is correct for BSCCO.

A curious point is that the melting line $H_m(T)$ slopes backwards, like the line of melting pressures $p_m(T)$ in the phase diagram of water. This negative slope, when combined with the thermodynamic Clausius-Clapeyron relation, requires that the solid phase actually becomes denser on melting. Zeldov *et al.* observe this effect, and their data imply an entropy change of about $0.5k_B$ per vortex per copper-oxide plane. The anomalous density change of H_2O on melting (which arises because hydrogen bonds lead to an unusually open crystal structure) means that the ice melts under pressure, as ice skaters and cross-country skiers know. The external field in superconductors acts like a 'pressure' forcing vortex lines into the sample, and it is worth understanding why the Abrikosov flux lattice also

melts when 'squeezed' by increasing the external field.

Although more detailed theories are available², a qualitative understanding results if thermal fluctuations in the crystal are modelled by a single vortex filament in the cage formed by the repulsive interactions with its neighbours⁹. Both the flexibility of the line and the range of interactions are important. The flexibility determines how far along the average field direction the line wanders before it is deflected back by the bars of its cage. The denser the vortex array, the more these collisions increase the entropic cost of the crystalline phase. When the vortex interactions are relatively long range (as is usually the case), squeezing triggers a melting transition when the density is high enough that the extra entropy of vortex braiding in an entangled flux liquid phase outweighs the potential energy cost. A density reduction on freezing might also be expected when flexible nematic polymers with low salt concentration crystallize into the hexagonal columnar phase. DNA molecules, for example, have interactions quite similar to vortices in

superconductors¹⁰. Similar physics causes quantum solids with long-range interactions to melt when squeezed, with zero-point energy replacing entropy in the above arguments.

Related arguments² show that the Abrikosov vortex crystal must also melt at very low densities and fields, along the re-entrant dashed curve shown in the figure. The physics of low-field melting, the field dependence of vortex configurations at very low temperatures and the behaviour above the high-field critical point are inviting subjects for future investigation.

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ARCHAEOLOGY

Drought and decline

Jeremy A. Sabloff

AROUND AD 800, something happened to the Classic Maya civilization. The relatively sudden decline seen at many sites, particularly in the southern lowlands (which lie in modern-day Guatemala, Mexico, Belize and Honduras), has garnered considerable scholarly and lay interest over the years¹. On page 391 of this issue, Hodell and co-workers² supply fresh evidence that may point the finger at climate changes in the region as one key factor in this decline.

In the past two decades, as archaeological field research in the Maya area has boomed and the deciphering of Maya hieroglyphic texts has generated new historical information, archaeological understanding of the 'collapse' of Classic Maya civilization has grown^{3–5}. Interrelated factors including population growth, environmental degradation and intercity conflict have been implicated in the process of decline. In addition, the concurrent florescence of cities in the northern lowlands such as Uxmal, Kabah, Sayil and Chichén Itzá and the lack of decline at certain sites in the southern lowlands with advantageous riverside locations can now be better appreciated, as these centres are situated either in zones with previously low populations or in locations that provided economic buffers against severe environmental strains. It also has become apparent that the exact

triggers, many of which have yet to be identified, for the collapse of individual cities may have varied within the overall matrix of demographic and environmental pressures.

The new research by Hodell, Curtis and Brenner², adds another important component to the model that archaeologists are currently building to help explain the upheavals of the late eighth century and early ninth century AD in the Maya lowlands. As some of the factors were present well before AD 800 and the ancient Maya suffered previous setbacks in their development⁶, one unanswered question has been why these factors had such a cataclysmic effect around AD 800. What Hodell *et al.* find, by examining sediment cores from Lake Chichancanab in the northern lowlands of the Yucatán peninsula (see map), is that a period of increased climatic dryness set in around AD 800.

To a civilization facing a number of stresses both internal and external, the scarcity of water could have greatly increased the vulnerability of numerous Classic Maya cities, especially in the southern lowlands where the strains were greatest. In particular, with the landscapes around many Maya centres already under heavy stress^{7,8}, the arrival of a drier regime would have exacerbated the perilous situation, ultimately causing the de-

mise of elite power, the abandonment of many urban centres, and important demographic and economic shifts from the southern to the northern lowlands. Continued dryness for two centuries, as the



The Yucatán peninsula showing the coring site (Lake Chichancanab) of Hodell *et al.*² and Maya sites mentioned in the text.

results of Hodell *et al.* suggest, might also have inhibited cultural and demographic recovery in the previously degraded parts of the southern lowlands.

The research by Hodell and colleagues contributes to scholarly attempts to understand why and how the Classic Maya successfully adapted to their tropical environment for so many centuries, overcoming numerous problems and reaching population levels that far exceed modern ones in the same area, and why they ultimately failed. Their research also raises an anthropological question that is relevant not only to the ancient Maya but to the contemporary world as well, and is certainly deserving of continued attention — namely, how severe do internal stresses in a civilization have to become before relatively minor climate shifts can trigger widespread cultural collapse? □

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Impact consensus emerges

Peter J. T. Leonard

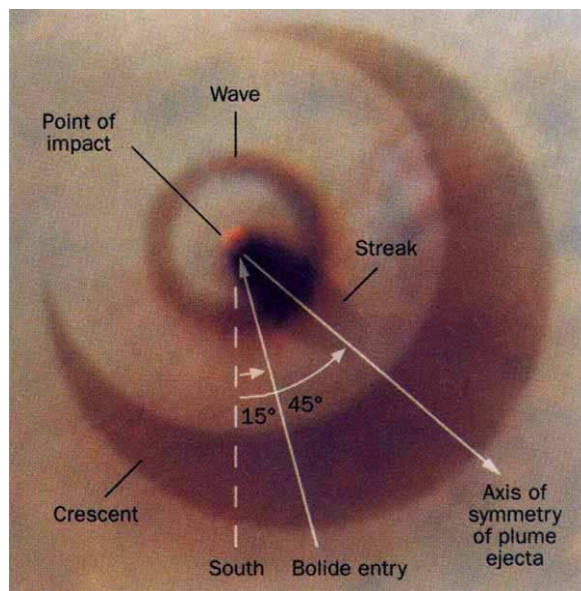
WHAT happened during the first hour after each fragment of comet P/Shoemaker–Levy 9 struck Jupiter in July 1994? A meeting last month on the comet impact* revealed a surprising convergence of opinion regarding observations that had everyone baffled just months ago. General agreement was reached about the nature of the various flashes seen during each impact, the explanation for the shapes and orientations of the dark spots observed at each impact site, and the size of the largest fragment. The meeting was a remarkable scientific show; it required a year of work by hundreds of talented scientists — and of course the collision of a comet with a major planet.

From a comparison of observers' notes during one of several subworkshops, a unified picture emerged for the light curve of a hypothetical impact as viewed from the Earth. First seen was a faint glow that slowly increased in brightness on a time-scale of tens of seconds, believed to be due to a large number of small meteors in the coma surrounding each fragment. The meteor shower was followed by a very sharp increase in brightness as the main part of the fragment entered the jovian atmosphere. This bolide phase lasted less than ten seconds and was directly observed by the Galileo spacecraft, which was 1.5 AU away from Jupiter at the time. During the impact of fragment W, the bolide was detected by the Hubble Space Telescope (HST), presumably in reflection off dust in the coma. A few tens of seconds later, a fireball exploded up the 'chimney' created in the atmosphere by the bolide. The fireball was initially hot enough to be self-luminous at optical wavelengths and was detected by the HST. Later, this plume was visible via reflected sunlight as it emerged from Jupiter's shadow.

The 'main event', which was so very prominent in the infrared, peaked ten minutes after impact. This was the 'splash' caused by the 'fallback' of the plume onto the jovian atmosphere. Some of this fallback 'bounced', and a second peak in thermal emission was seen during a second splash ten minutes after the main event. Modelling of the emission from the bounces neatly illustrates a 10-minute periodicity (Drake Deming, NASA Goddard Space Flight Center).

The most notable consequence of the impacts was the dark features that resulted from the fallback of the plume onto the atmosphere. This dark material has come to be known technically as the

'brown stuff'. Extending to the southeast (in a jovigraphic sense) from each of the impact sites was a very dark 'streak', lying on the axis of symmetry of a much larger but lighter coloured crescent. (The 'wave' seen by the HST to be moving radially away from several of the impact sites will be discussed below.) It was perplexing that the axis of symmetry of the streak and crescent was twisted 45° east of south, whereas the chimney that shot out the plume was orientated 15° east of south,



The geometry of the impact site of a major fragment of Comet Shoemaker–Levy 9. The difference between the angle of bolide entry and the axis of symmetry of the plume ejecta is due to Coriolis deflection during a 10-minute ballistic flight followed by a 30-minute horizontal flow.

and the Coriolis deflection during the 10-minute ballistic flight of the ejecta was only an additional 10°. Kevin Zahnle (NASA Ames Research Center) and Gene Shoemaker (US Geological Survey) argued convincingly that the remaining 20° of rotation is the result of a Coriolis deflection during 30 minutes of horizontal flow as the plume ejecta bounced across the jovian atmosphere. Unfortunately, the nature of the ubiquitous brown stuff remains obscure. Among the candidates are grains of silicates from the impacting body coated with poly-HCN. The latter substance mixed with water readily produces amino acids, the building blocks of life (Clifford Matthews, Univ. Illinois).

The most complete picture of the impacts arises if the diameters of the largest fragments were only 0.7 km. There are several arguments in favour of this interpretation: the dimmer-than-expected fireballs, the lack of seismic waves, the lack of atmospheric disturbances at millimetre

and centimetre wavelengths, the lack of chemistry consistent with dredged-up water, the estimated total mass of dust, and the meagre evidence for impacts due to small, previously unknown fragments (Mordecai Mark MacLow, Univ. Chicago). The sub-kilometre diameter is consistent with tidal disruption models of the parent body, which point to a comet with a diameter of only 1.5 km and a density of 0.5 g cm⁻² (E. Asphaug and W. Benz/J. C. Solem *Nature* **370**, 120–122/349–351; 1994). In addition, one can explain the almost constant plume heights (3,000 ± 500 km) from impacts of fragments of quite different mass only if the largest fragments did not penetrate far into the troposphere (Zahnle).

There are still a few who would prefer the largest fragments of Comet Shoemaker–Levy 9 to have been 2 km in diameter rather than less than a kilometre. David Crawford (Sandia Labs) asserts that comparisons of his hydrodynamic models with observation favour the larger size. Andy Ingersoll (California Inst. Technol.) concludes that the wave detected by HST moving away from the impact sites at 450 m s⁻¹ can be explained as the result of a tropospheric gravity wave that travels horizontally through the water vapour layer in the jovian atmosphere (A. P. Ingersoll and H. Kanamori, *Nature* **374**, 706–708; 1995). A small dip in stratospheric temperature occurs as the wave passes by, which causes the 'brown stuff' to condense

and then evaporate. Ingersoll's wave requires fragment sizes consistent with Crawford's estimates in order to deposit at least 10²⁷ erg deep in the troposphere.

Finally, although there were several comments during the meeting that it would be nice to have a second comet hit Jupiter to firm up the observations, another impact is not likely to happen any time soon. Gene Shoemaker estimates that, on average, a 1.5-km-diameter comet is captured and tidally disrupted by Jupiter and strikes the planet once every 2,000 years. He added that for the impacts to happen as they did — after the repair of HST, when the Galileo spacecraft was suitably placed, during the era of very efficient infrared detectors, and while the United States government is still investing in basic research — was a miracle indeed.

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*IAU Colloquium 156: The Collision of Comet P/Shoemaker–Levy 9 and Jupiter, Baltimore, Maryland, USA, 9–12 May 1995.

Dymer, dymer binding tight

Ben Luisi and Leonard Freedman

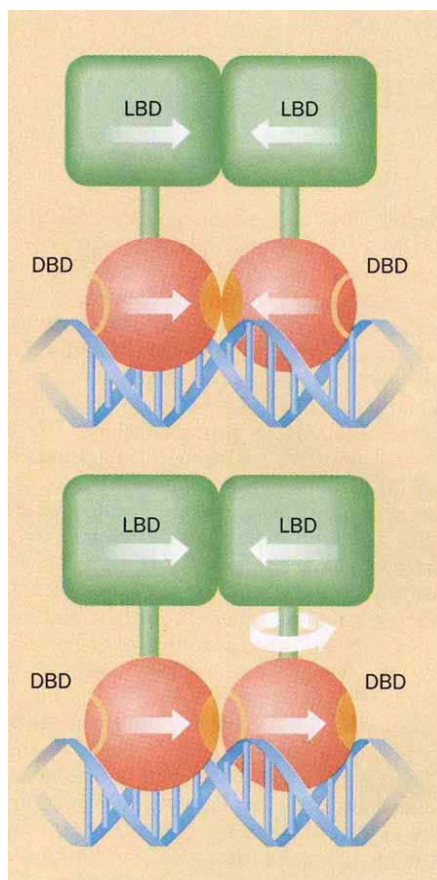
EARLY in the evolution of multicellular organisms, a fortuitous accident enabled tissues to communicate over long distances. The event, a fusion of two genes encoding small proteins binding DNA and lipophilic molecules, respectively, permitted diffusible signals to have a direct effect on gene expression. So successful was the modular molecular design that it has been maintained throughout the animal kingdom, and is seen in the conserved domains of a large family of ligand-activated transcription factors known as the hormone or nuclear receptors. The DNA-binding domains of several family members have been characterized structurally, revealing a common, metal-bearing fold¹.

With the report of Bourguet *et al.* on page 377 of this issue², the ligand-binding domain (hereafter LBD) is now also set on a stereochemical foundation. The authors present the crystal structure of the apo-LBD from the human receptor for 9-*cis* retinoic acid (α -isoform, or RXR- α), and it seems likely that its fold, like that of the DNA-binding domain, is common throughout the receptor family. This study also provides the first structural glimpse at the mysterious transcriptional activation domain which resides in the LBD carboxy terminus.

Many of the nuclear receptors dimerize through the LBD. The RXR- α receptor appears to associate avidly not only with itself, but also with other receptors, including those for vitamin D, thyroid hormone and all-*trans* retinoic acid. This association confers an enhanced affinity for a distinct DNA target on each heterodimer³, permitting the 'cross-talk' of different ligands and potentially baroque physiological effects.

Bourguet and colleagues' X-ray structure of the RXR- α LBD shows that the dimer interface coincides with a crystallographic symmetry element and is dyad-symmetrical (see figure). It had been noted earlier that the LBD bears a conserved heptad repeat of leucine and other hydrophobic residues, and in 1990 Forman and Samuels⁴ put forward the inspired hypothesis that this motif forms a coiled-coil dimerization surface in analogy with the interface found in the leucine zipper and helix-loop-helix transcription factors. It turns out that in the LBD crystal structure, the dimer interface is composed of packed helices and the heptad residues do not directly contribute to the interface. Instead, these residues are interspersed throughout the structure to provide architectural support. As predicted⁵, two helices from each monomer participate in the dimer interface, resulting in a packed helical cluster.

Judging from the conservation of the hydrophobic scaffold, it seems that the LBD architecture is conserved, but details of the dimer interface are likely to vary. This is expected because certain receptors, such as those binding steroids, seem only to form homodimers and do not cross-talk. Variability at the dimer interface may therefore define partner choice and, consequently, result in either tight compartmentalization or complex cross-talk in the effects of various ligands. This



Symmetry of ligand-binding domain and response elements for nuclear receptors. The dyad symmetry of the LBD dimer interface stands in contrast to the translational symmetry of the nuclear receptors' DNA targets, which are either direct or inverted repeats^{1,3}. Steroid receptors form homodimers and bind inverted repeats whereas nuclear receptors, which form heterodimers, recognize direct repeats. The DNA-binding domain can mediate recognition of these repeats through protein-protein interactions^{1,14}. The match of dimeric receptor to directly repeating element can only be explained if the peptide connection between the DNA- and ligand-binding domains is flexible, like a telephone cord. The LBD dimer therefore serves as a physical tether. DNA binding benefits from this design, because the tether decreases the entropic cost of binding and contributes to an implicit cooperativity.

type of functional discrimination is analogous to the assembly equilibrium of Jun, Fos and other leucine-zipper transcription factors.

Ligand binding is required by most nuclear receptors for modulating gene expression, but the RXR- α LBD crystal structure has been determined in the absence of 9-*cis* retinoic acid, so it is not clear how the ligand acts structurally. Ligand binding affects dimer/monomer equilibrium in certain receptors⁶; conversely, dimerization can affect ligand affinity⁷. These observations indicate that the ligand-binding cavity and the dimer interface are communicating — which, conceivably, could result in quaternary structural change in the dimer. There is spectroscopic evidence for minor secondary structural changes with ligand binding in some receptors⁸, and the ligand can also decrease protease sensitivity⁹. The ligand-binding cavity can be readily modelled to fit 9-*cis* retinoic acid with little conformational adjustment², so the structural responses to ligand may be triggered by very subtle changes in the pocket.

The LBD also encompasses the transcriptional activation domain, called AF-2, which interacts with auxiliary proteins¹⁰ to communicate with the RNA polymerase transcription complex. AF-2 is part of an α -helix and is not disordered as is sometimes expected of activation domains. The helix is partly exposed on the surface of the receptor, but some of its buried residues have been genetically defined^{11,12} as mediating interactions with co-adaptors². Ligand binding, which triggers transcriptional activation, presumably affects the presentation of AF-2. Two mechanisms can be envisaged: first, that tertiary and secondary structural alterations unveil the hidden AF-2 residues; second, that subunit rearrangements occur through a quaternary transition.

If substantiated, the conformational change of AF-2 may explain the stereochemical puzzle of how some regulatory cofactors interact promiscuously, yet with presumed specificity, with many dissimilar proteins. Perhaps the mechanism involves refolding of secondary structure to yield a variety of stable complexes (there is analogous structural plasticity in other regulatory systems¹³). Furthermore, if co-adaptors recognize the spacing and orientation of the dyad-related activation helices, then a simple rotation of the two LBDs brought about by ligand-induced quaternary change could also act as a regulatory switch. Subtle differences in the rotation or fold could effect the accommodation of distinct cell-specific or developmental factors.

Studies of the DNA-binding domain have revealed how a few amino acids can generate the rich diversity of DNA recog-

nition among nuclear receptors^{1,14}. Judging from the pattern of sequence conservation, it seems that the functional repertoire of the LBD is similarly generated from a few crucial residues that affect specificity for ligand, dimerization and auxiliary proteins. Further stereochemical characterization of RXR- α and other nuclear

receptors should continue burning bright to illuminate their functions. □

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(which lack licensing factor) to the licensing factor present in the soluble fraction of *Xenopus* egg extracts permits the G2 nuclei to reinitiate replication¹⁷.

Experiments using these assays have now permitted the three laboratories to conclude that a complex of MCM proteins is an essential component of licensing factor. Chong *et al.*¹ show that licensing-factor activity can be resolved into two essential components, M and B, neither of which is sufficient by itself for licensing. The M component has been purified through four chromatographic steps by assaying for activity in the presence of a constant amount of crude B component. Purified M component consists of a complex of several proteins, all of which appear to be members of the MCM family¹.

Madine *et al.*² and Kubota *et al.*³ demonstrate that licensing-factor activity can be removed from interphase *Xenopus* extracts with antibodies specific for *Xenopus* MCM3 and can then be restored by addition of the proteins released from the antibodies. Interestingly, these proteins are not just MCM3 but include other family members, similar to the complex of several MCM proteins in the M component purified by Chong *et al.*¹ Evidence that vertebrate MCM proteins act together as a complex, or perhaps as several complexes, comes from other research groups^{13,18} and is in agreement with previously demonstrated genetic interactions between MCM proteins in yeast⁵.

Consistent with Blow and Laskey's original definition of the hypothetical licensing factor¹, restoration of the MCM protein complex to extracts that are immuno-depleted or treated with protein-kinase inhibitor permits just a single round of replication¹⁻³; and immunological studies employing antibodies against *Xenopus* MCM3 reveal that MCM3 binds

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CELL CYCLE

A licence to replicate

Joel A. Huberman

THREE papers — two appearing on pages 418¹ and 421² of this issue, the other in *Cell*³ — mark the beginning of a new era in the study of so-called replication licensing factor. They show unequivocally that members of a particular protein family are components of licensing factor, and thereby turn a hypothetical principle into a much more tractable subject for research.

Chromosomal DNA replication in eukaryotic cells is limited to a single round per cell cycle, and the question is how the process is restricted to one round and one round only. Seven years ago, based on studies of DNA replication in extracts from eggs of the amphibian, *Xenopus laevis*, Blow and Laskey⁴ proposed that the limitation is accomplished by a replication licensing factor. Its molecular nature was unknown, but Blow and Laskey pointed out that the factor must gain access to chromosomes during mitosis, must be essential for replication and must be inactivated by the replication process. So during a single round of replication all licensing factor would be inactivated, preventing initiation of new replication until after mitosis.

Independently, the laboratories of Tye and Botstein searched for budding-yeast genes encoding proteins essential for initiation of DNA replication at replication origins (reviewed in ref. 5). Their search led to the identification of a family of related, genetically interacting proteins, now usually called the MCM family (for minichromosome maintenance, because mutant proteins selectively destabilize replication-origin-dependent circular minichromosomes in yeast). These groups^{6,7} then found that the MCM2, MCM3 and MCM5 (also known as CDC46) proteins are located inside yeast nuclei immediately after mitosis, but are lost from nuclei once DNA synthesis

begins. This behaviour suggested that the MCM proteins might correspond to at least a component of licensing factor.

Since then, additional evidence has accumulated that the MCM proteins may be related to licensing factor. Mammalian homologues of some of them have been identified⁸⁻¹²; and antibodies against vertebrate MCM proteins block DNA synthesis^{10,11,13}. Although mammalian MCM proteins, unlike those of yeast, are present in nuclei during all of interphase, they are tightly bound in the nucleus only before DNA synthesis (G1 phase). As would be expected for licensing factor, they are released from their tight binding once DNA synthesis begins (S phase) and do not rebind after DNA synthesis is complete (G2 phase)^{11,14}.

Meanwhile, the three laboratories now reporting, those of Blow¹, Laskey² and Takisawa³, were developing biochemical assays for licensing factor¹⁵⁻¹⁷. One assay takes advantage of the fact that treatment of mitotic egg extracts with protein-kinase inhibitors prevents the activation of licensing factor during the subsequent transition to interphase, thus preventing initiation of DNA replication in the resulting interphase extract^{15,16}. However, when demembrated *Xenopus* sperm nuclei (sperm chromatin) are briefly exposed to untreated interphase extract, they can be assembled into nuclei and can undergo a single round of replication in extracts treated with protein-kinase inhibitor. So, by incubating sperm chromatin with fractions from untreated extract and then testing for replication competence in treated extract, it should be possible to identify the fractions of untreated extract that contain licensing factor^{15,16}. The second assay is based on the observation that exposure of permeabilized mammalian G2 phase nuclei

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to sperm chromatin at the end of mitosis, remains bound during G1, and is released from the chromatin during S phase¹⁻³.

Now that it is clear that replication licensing factor is an actual complex of proteins that can be studied biochemically and genetically, more detailed questions can be posed. What proteins are present in the B component? What is the mechanism of action of licensing factor? Is there just a

single licensing factor in each species, or do different tissues employ licensing factors containing different members of the MCM family? These and other issues should be resolved before too long. □

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ELECTRON CRYSTALLOGRAPHY

Taxol found on tubulin

Lee Makowski

TEN years ago the best electron microscope images of globular proteins were virtually all little more than shapeless blobs. These days, as a consequence of relentless technical advance, electron crystallography is capable of producing images at resolutions close to those attained by X-ray crystallography or multidimensional NMR. New images of taxol*-bound tubulin sheets, presented by Nogales *et al.* on page 424 of this issue¹, now show that electron crystallography can be used to image the binding of a drug molecule to its biological target protein.

Tubulins have a central role in eukaryotic biology. In solution they occur as heterodimers composed of one copy each of α - and β -tubulin, and they are the principal component of the microtubules that form the framework of molecular machines required for movement of flagella, organelles and chromosomes. Their participation in chromosome movement during mitosis makes tubulins attractive targets for antitumour drugs.

Taxol and related taxane anticancer agents are among the most promising chemotherapeutic agents developed during the past decade². They suppress the microtubule dynamics required for chromosome movement during mitosis, stalling cell division at the metaphase/anaphase transition. Taxol, a complex diterpenoid containing the taxane ring system, a four-membered oxetane ring and an ester side chain, was first isolated³ from the bark of Pacific yew trees in 1967 and chemically synthesized only last year^{4,5}. It has been approved by the US Food and Drug Administration for use in the treatment of refractory ovarian cancer and metastatic breast cancer, and is under intensive study with the aim of designing more potent anticancer drugs.

Standard operating procedure in structure-based drug design would be to crystallize tubulin in the presence of taxol and determine the high-resolution structure. The details of the taxol-tubulin interaction could then be used as a guide

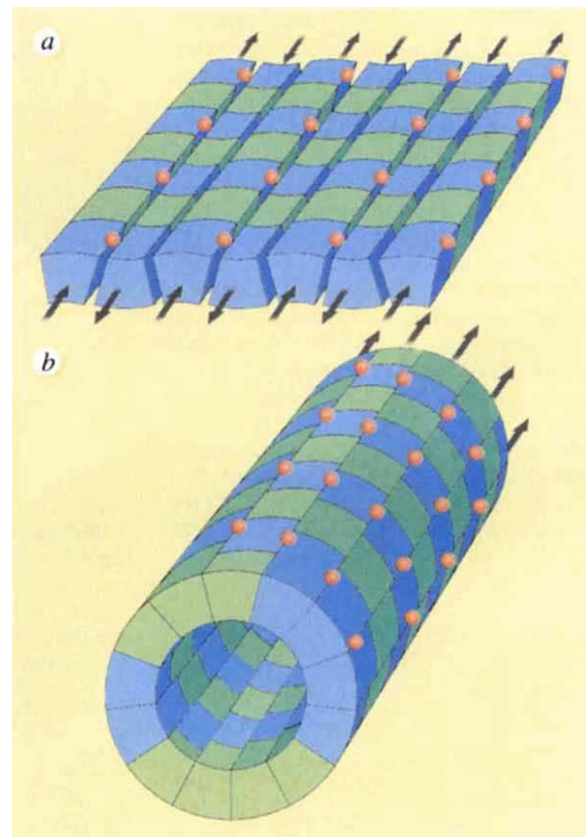
to design taxol analogues that would bind more tightly to tubulin and have improved pharmacological properties. Unfortunately, all attempts to produce usable crystals of tubulin (and there have been a great many) have failed.

Enter electron microscopy. Nearly 20 years ago, two-dimensional tubulin crystals were formed by binding zinc^{6,7}.

The zinc sheets are highly ordered and contain more subunits than microtubules, making them more suitable for high-resolution electron crystallography than the microtubules themselves. The arrangement of tubulin in these zinc-induced sheets bears a simple relationship to that in intact microtubules. As shown in the figure, the protofilaments of alternating α - and β -tubulin are preserved in the two structures, but the interactions between protofilaments are very different. Electron microscopy of negatively stained zinc sheets was originally used to construct a 20 Å resolution image of a microtubule⁸; that map suggested a general shape for the tubulin monomers and demonstrated that the structures of α - and β -tubulin are different in detail. The path from the 20 Å map to the 6.5 Å map presented by Nogales *et al.* has been made possible by extraordinary developments in electron microscope techniques and technology^{9,10}. The images created by electron microscopy are no longer simply micrographs; rather they are three-dimensional density distributions derived from dozens or even hundreds of micrographs.

The studies of Nogales *et al.* have yielded tubulin images of unprecedented detail. The external surface of tubulin appears to be quite smooth, in sharp contrast to the interior surface from which strands of β -sheet appear to form ragged projections. An α -helix, presumably near the carboxy terminus, has been identified. A β -sheet seems to make up a substantial part of the axial contacts between α - and β -tubulin molecules within a single protofilament. The complete chain tracing is not yet defined because of the limited, anisotropic resolution of the study. Resolution is lower in the direction perpendicular to the sheets, because of limitations in the tilt angle of the specimen relative to the electron beam which tends to elongate structural features in the direction perpendicular to the sheets. The 3.8 Å resolution difference map accurately defines the taxol binding site in projection — that, and the fact that the taxol binding site is accessible to the outside surface of microtubules¹¹, defines the position of bound taxol to within a few angstroms.

Taxol blocks mitosis by stabilizing mic-



The organization of tubulin dimers in the zinc sheets described by Nogales *et al.*¹ (a) has a simple relationship to that in intact microtubules (b). Whereas the protofilaments in a microtubule are parallel to one another, the protofilaments in zinc sheets are antiparallel and their external surfaces alternate, being oriented 'up' then 'down' relative to the plane of the sheet. Consequently, although the interactions stabilized by taxol in zinc sheets and in microtubules are both between pairs of β -tubulins, the contacts in these two structures are completely different. The taxol binding sites are in red; the β -tubulins are blue and the α -tubulins green.

*The word "Taxol" is a registered trademark of Bristol-Myers Squibb, which offers the term paclitaxel instead.

rototubes and promoting the polymerization of tubulin¹². It does so by binding near the amino terminus of β -tubulin, as shown by photoaffinity labelling studies¹³ that demonstrated the crosslinking of the taxol sidechain to a peptide consisting of the first 31 amino acids of β -tubulin. This region of tubulin is also involved in GTP binding¹⁴, and the action of chemically unrelated antimitotic drugs¹⁵ that act by inhibiting tubulin polymerization. Thus taxol binds to a portion of tubulin that acts as a control centre for tubulin polymerization.

Induction of tubulin polymerization by taxol could involve changes in the axial interactions of tubulin along protofilaments or in the radial interactions of the protofilaments with one another. The location of the taxol binding site between protofilaments in the zinc sheets indicates that inter-protofilament interactions are the controlling elements. The possibility that taxol 'bridges' two protofilaments in microtubules cannot be directly inferred from this result. As shown in the figure, the interactions among protofilaments in these sheets are very different from those in intact microtubules. Taxol stabilizes zinc sheets¹ and promotes polymerization of microtubules with a variable number of protofilaments¹². At higher resolution, the mechanism by which taxol affects the polymerization of tubulin may become clear.

It is simply a matter of time and hard work before this approach will yield a higher-resolution image of tubulin and its interactions with taxol. This is good news for anyone interested in tubulin, or in any macromolecular system that is not conducive to X-ray crystallographic analysis. Drug designers should take note. $\square$

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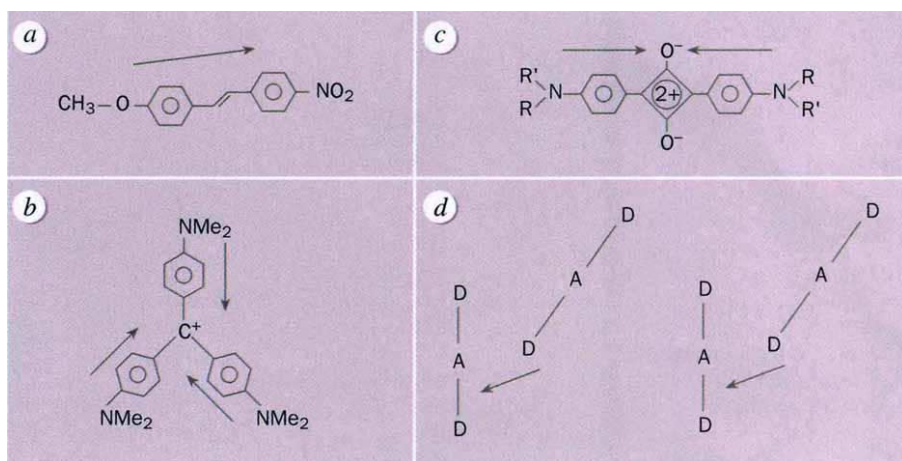
Heresy in the world of organic nonlinear optics

J. L. Brédas and F. Meyers

HERESY — that is how Ashwell and colleagues characterize the approach they follow in the work reported on page 385 of this issue¹. These authors describe a strong second-order nonlinear optical signal in an ultra-thin film built from centrosymmetric squaraine dyes. As significant even-order optical effects only arise in noncentrosymmetric media, the new results open up an entirely new line of thinking in this technologically important field.

Nonlinear optical (NLO) phenomena are at the heart of modern communications systems in which optical signals need to be transmitted, processed and stored.

starts by emphasizing the overall noncentrosymmetry required for such processes to take place in any significant way. So, not surprisingly, the design of new organic systems has focused on two things. First, the consideration of noncentrosymmetric molecules: these are mostly of the dipolar type, presenting a one-dimensional charge-transfer axis. Prototypical examples are donor-acceptor polyenes² or stilbenes, such as the 4-methoxy-4'-nitrostilbene molecule (part *a* of the figure). More recently, studies have also been devoted to octupolar-type molecules³ that have no dipole moment in the ground state and are noncentro-



a, b, Molecular structures of prototypical noncentrosymmetric organic molecules considered for second-order nonlinear optical processes (*a*, methoxynitrostilbene; *b*, crystal violet cation). *c*, Molecular structure of the centrosymmetric squaraine dye investigated by Ashwell *et al.*¹. *d*, Possible dimer arrangement and packing of the squaraine dyes.

They originate in the fact that the polarization response of a medium can become highly nonlinear when subject to the intense electric field of a laser beam. In an organic medium, at the molecular level, the induced dipole moment can then be cast in the form

$$\mu_{\text{ind}} = \alpha \cdot E + \frac{1}{2} \beta \cdot E E + \frac{1}{6} \gamma \cdot E E E + \dots$$

where α , β and γ denote the first-, second- and third-order molecular polarizabilities, respectively, and E is the electric field associated with the light. Communications systems exploit second-order NLO effects such as the modulation and switching of an optical signal by an external electric field (Pockels or linear electro-optic effect, leading to electro-optic modulators) or the doubling of light frequency (second-harmonic generation, SHG).

Almost any review paper or lecture dealing with even-order NLO effects

symmetric; these are molecules with, for instance, three-fold or tetrahedral symmetry and multiple charge-transfer axes, such as the crystal violet cation (part *b* of the figure). Second, strategies have been used to preserve the absence of centrosymmetry at the macroscopic level; these include alignment of the NLO chromophores under electric field (poling)^{4,5}, exploitation of chirality^{6,7} or controlled film formation via Langmuir-Blodgett deposition techniques^{8,9}.

In view of such symmetry requirements, the approach of Ashwell *et al.*¹ does indeed seem heretical. They consider a squaraine dye molecule that is centrosymmetric with a one-dimensional, bidirectional charge-transfer axis (part *c* of the figure) and build from it a Langmuir-Blodgett monolayer film. If the individuality of the molecular entities was preserved in the film, no significant second-harmonic signal could be expected — but in fact, the SHG signal measured by

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Ashwell *et al.* is among the highest ever reported for a Langmuir–Blodgett monolayer. According to the authors, the pressure applied to the dye molecules before film deposition induces intermolecular interactions between the acceptor part of one molecule and the donor part of an adjacent molecule, resulting in dimer formation and intermolecular charge transfer; a rough description of a possible dimer structure is given in part *d* of the figure. In this case, noncentrosymmetric dimers become the molecular building blocks and SHG could be observed provided the dimers pack in a noncentrosymmetric fashion.

Besides the novelty of starting with centrosymmetric chromophores to build a second-order active NLO material, another fascinating aspect of the work of Ashwell *et al.* is to exploit intermolecular charge transfer to provide a very large optical nonlinearity¹⁰. Until now, inter-

molecular interactions had mostly been used to try to stabilize a noncentrosymmetric bulk arrangement⁷, not to provoke noncentrosymmetry at the molecular level.

Much work remains to be done to assess more accurately the structure of the Langmuir–Blodgett films reported in the new paper¹. One important aspect is to work out how the pressure applied to the floating monolayer affects the resulting structure. Control of this process is essential for the development of other potentially interesting systems of the same kind. Another obvious task will be to evaluate theoretically the packing of such molecules, for instance via molecular mechanics techniques; and on that basis, to calculate via quantum-chemical methods the hyperpolarizability contributions from intermolecular charge transfer. Whether or not the results of Ashwell and colleagues bring any direct benefits to non-

linear optical applications, it is clear that they are most exciting from a conceptual standpoint. □

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MATERIALS SCIENCE

Epitaxy keeps rolling along

Robert W. Cahn

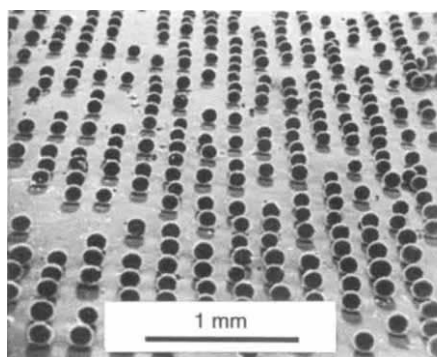
THE oblique approach — creeping up on an obstinate problem when it is not looking, so to speak — is apt not only to be elegant, but also to carry conviction in situations where that is hard to come by. Just such an approach is to be found in a study, by Shirokoff and Erb¹, of a puzzling aspect of epitaxy.

Epitaxy is the deposition, typically by evaporation, of one chemical species on a single crystal of another, in such a way that the deposited film has a specific and reproducible crystal orientation relative to that of the substrate; it is a matter of fit and interaction between the two crystal lattices. Epitaxy is of great scientific and industrial interest, particularly in microelectronics. The puzzling aspect referred to is that epitaxial relationships can in certain instances be achieved when an amorphous film separates the substrate and the deposit; this might be termed indirect epitaxy. This seemed inherently so improbable that for years many scientists have refused to take the experimental evidence at face value. For instance, some have suggested that the amorphous films in these experiments contain invisible pinholes through which deposit and substrate were able to interact directly. The new work was undertaken to test these suspicions.

The original observations on indirect epitaxy were due to Distler, who worked in the renowned Institute of Crystallography in Moscow, over the period 1966 to 1976 (see earlier News and Views article²). A further unexpected feature of Distler's observations was that the initial

deposit islands were often unoriented and only later rotated into epitaxial orientation, before they became subsumed in a continuous film. The process, which came to be termed postnucleation, became an intensive subject of research soon afterwards³.

Distler's last published paper on indirect nucleation and postnucleation⁴ was concerned with deposits of silver chloride on (ferroelectric) triglycine sulphate, with and without amorphous selenium layers, thick enough to be continuous, separating the two species. Epitaxy was maintained when the film was present, and a diverse range of considerations were presented in support of the notion that electric fields from the ferroelectric domains of the substrate, acting through the selenium film when present, imposed the epitaxial orientation on the silver chloride⁵. It makes a convincing story.



The indirect approach referred to in my opening sentence is the small-sphere sintering approach first suggested by Shewmon in 1965 and put into effect by Herrmann, Gleiter and co-workers 10 years later⁶. The issue that troubled Shewmon at the time was the absence of evidence that the plot of specific energy against angle of misorientation in inter-crystalline boundaries in metals had local minima or 'cusps', although theory predicted these. He suggested sintering pairs of small single-crystal spheres together in random orientations and waiting for them to rearrange themselves (that is, roll) so that the grain boundary between them occupied a local minimum in energy.

Herrmann *et al.* put this clever idea into effect by scattering thousands of single-crystal copper spheres about 100 µm in diameter (made simply by melting small copper particles) onto a single-crystal copper plate and annealing them to sinter the spheres to the plate (Fig. 1). The statistically preferred orientation of the spheres was determined by X-ray diffraction. In this way, the expected cusps in the energy plot were indeed found; the technique was

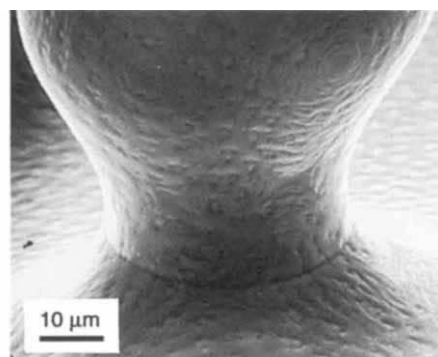


FIG. 1 Scanning electron micrographs of an array of copper spheres sintered to a copper plate, and of a sphere in close-up. A grain boundary can be seen in the sintered neck. (Courtesy H. Gleiter.)

later extended in various ways, for instance to test the effect of solute on the energy plots⁷.

Shirokoff and Erb¹ studied gold and silver on sodium chloride substrates (classic epitactic combinations), and also on silicon substrates, using evaporated amor-

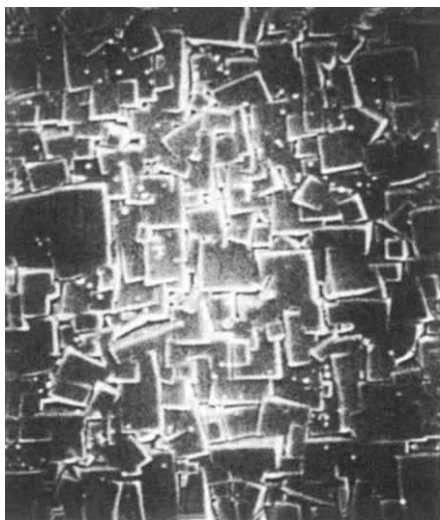


FIG. 2 Approximate epitaxy of diamond deposited on silicon. From ref. 12 (courtesy S. J. Bull).

phous carbon separators in the former case, and amorphous silica films grown *in situ* in the latter. The metals were evaporated into position and then melted there to form small spheres, which were sintered into position. The same X-ray approach as before was used to test for the nature and uniformity of epitaxy. The experiment therefore amounted to searching for cusps in the plot of specific energy against misorientation for phase interfaces (as distinct from grain boundaries in the original experiments). Erb used to be a collaborator of Gleiter, so the approach has a direct pedigree. The outcome, in brief, was that epitaxy was maintained in the presence of amorphous separators as thick as 200 Å, though the thicker the amorphous separator, the less distinct, statistically, was the orientation relationship.

The details of the specific orientations do not matter here; there are several distinct relationships between any one substrate/deposit pair. What is important is that the orientation relationships fell into two categories: those that are sensitive to the presence of separator layers, and those that are not.

The experiment puts an end to the suspicion that Distler's findings were due to pinholes, for three reasons: if there had been a few of these, only those microspheres that happened to coincide with a pinhole would be affected; the division of orientations into two families was analysed to show that this fact was inconsistent with direct substrate/deposit contact; and a Au-Si eutectic was observed when

Au and Si were in direct contact but not when a thin SiO₂ film was present.

Shirokoff and Erb go on to say that it is now high time to attempt to model how epitaxy can in fact work through the separator films. It is hard to see how a Distler-type electric field could achieve this effect when the deposit is a metal.

The role of amorphous separators in permitting (or even fostering) epitaxy has surfaced in the past few years in another connection: making thin diamond films on suitable substrates by thermally induced reaction of gaseous methane with hydrogen. One development of this technique is to attempt to make single-crystal diamond films for microelectronics, using a silicon single-crystal substrate: silicon and diamond share the same crystal structure and although they have very different lattice parameters, approximate epitaxy can be achieved⁸⁻¹⁰; the result is a polycrystalline film with nearly parallel orientations. It is necessary to apply an electric bias between the substrate holder and the plasma above the substrate, and to permit the favoured orientations to grow over a period of hours at the expense of less favoured orientations (Fig. 2).

These papers, but especially those by Stoner's group, claim that an essential part of the epitactic nucleation of diamond grains on silicon is the formation of thin amorphous layers of silicon carbide, which have to act as intermediaries. The assertion has this year been queried by Yang *et al.*¹¹ who present evidence, in the form of high-resolution electron micrographs of a cross-section of a diamond deposit on silicon, that the amorphous silicon carbide layer has pinholes which permit direct contact between diamond and silicon in a few locations. This takes us back to the sceptics who contemplated Distler's surprising results. There is indeed nothing new under the Sun. □

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Personal décor

MANY cosmetic treatments claim to sink into the skin, with flattering, even dramatic, results. The only one which actually does so is tattooing. Painfully, point by point, it implants a permanent image under the subject's skin. Daedalus is now improving the process.

He is combining two modern inventions, the spray immunizer and the ink-jet printer. The spray immunizer fires a shot of vaccine at the skin with such force that it penetrates the epidermis and is deposited just under the skin. Unlike conventional needle injection, the process is rapid and does not hurt. The ink-jet printer, of course, builds an image from tiny ink drops ejected under computer control. DREADCO engineers are now combining the two into a computer-controlled 'Skinwriter'. Its flexible graphics software can optimize any image on screen; its contour-following print head, mounted on an articulated arm, can transfer that image in full colour and on any scale to any part of the customer's anatomy.

The slow and painful process of traditional tattooing will be replaced by a swift, painless scan taking less than a minute. But the DREADCO Skinwriter is not confined to the limited market for decorative dragons, hearts and roses. Daedalus hopes that it will transform conventional cosmetics as well.

The thinning of the ozone layer poses a worldwide dermatological threat. The Skinwriter could literally save our skins by implanting the lighter of us with a permanent, ultraviolet-absorbing tan. The usual brownish colour should be popular; but for those who wish to remain pale and interesting, a pure colourless ultraviolet absorber, essentially a lifelong sunblock, could be implanted.

More complex treatments would be just as simple. Pallid beauties could have the roses permanently restored to their cheeks, and would-be tough guys could be given an ingrained 5 o'clock shadow. Even wrinkles might be countered. The great ladies of the past enhanced their legendary complexions with arsenic. Daedalus reckons that it combined with the sulphhydryl groups of their dermal collagen, preventing these groups from crosslinking to each other to embrittle and wrinkle the skin. An arsenical Skinwriting ink could do this job even better. Fortunately, the intradermal region cannot easily clear away foreign material (which is why tattoos are stable). Firmly bound to the local collagen, the arsenic could not wander off into the body and cause trouble elsewhere. The secret of eternal youth may be a light implantation of arsenic. David Jones

Thyroid cancer in the Ukraine

SIR — When a serious accident occurs in a nuclear power station, fallout from radioactive iodine (mainly ^{131}I) is often one of the main sources of human exposure to ionizing radiation. Radioactive iodine may be taken into the body by inhalation or in food, a major source being milk, and is concentrated in the thyroid gland. In May–June 1986, several weeks after the Chernobyl accident on 26 April 1986, measurements of thyroid content of ^{131}I were made for 150,000 people in the Ukraine, including 110,000 children aged 0–18 years at that time¹. Because the thyroid gland is small in children and they drink more milk than adults, children were estimated to have received doses that were on average three times higher than in adults. The collective dose to children aged 0–18 in the entire Ukraine was estimated to be 400,000 person-Gy. On the basis of these estimates it was predicted that the accident might result in a discernible increase in thyroid cancer^{1,2}.

After the accident, the Ukrainian Research Institute of Endocrinology and Metabolism set up a register of thyroid cancer, receiving notification of all patients treated for thyroid cancer at hospitals throughout the Ukraine. Up to the end of 1993, 418 thyroid cancers have been reported in children and young adults who were aged 0–18 at the time of the accident: among them, 170 were aged 0–14 at the time their thyroid cancer was diagnosed and 248 were aged 15 or older. For 111 patients treated in the clinic of the Ukrainian Research Institute of Endocrinology and Metabolism, diagnoses were verified by four independent experienced histopathologists: 107 (96%) were papillary carcinomas, 2 (2%) were anaplastic, one (1%) was follicular and one (1%) was medullary.

The table shows the number of children aged 0–14 diagnosed with thyroid cancer in the Ukraine each year from 1986 to 1993 and the annual incidence rate per million. Incidence rates were fairly steady for the first 3 years, at around 0.7 per million per year, but have increased since 1989, the rate in 1993 being about five times higher than in 1986. (We are preparing a paper giving the clinical and other details of these cases.) Only two of the children with thyroid cancer were born after 1986, equivalent to an incidence rate of less than 1 per million per year in children born after the accident.

The increase in incidence of thyroid cancer in children in the whole of the Ukraine masks larger increases in the most heavily contaminated areas. The figure shows seven zones within the Ukraine divided according to estimated average thyroid dose in children. The legend gives the median dose to the thyroid and the annual incidence rate in children aged 0–18 at the time of the accident who were diagnosed with thyroid cancer in 1990–92. Among the 14,580 children aged 0–18 at the time of the accident who were living in Pripjat, a town 3.5 km from the Chernobyl plant, six have been diagnosed with thyroid cancer in 1990–92, corresponding to an annual incidence rate of 137 per million. Thus, the estimated average thyroid dose in children varied by several orders of magnitude within the Ukraine and there is a more than 30-fold gradient in thyroid cancer incidence rates in the country, corresponding to the gradient in thyroid doses from ^{131}I .

A similar increase in the incidence of thyroid cancer has been reported from Belarus³; the possibility was then raised that concern about the possible effects of the Chernobyl accident on thyroid cancer might have resulted in increased frequen-

cy and intensity of examinations of the thyroid in children and that cancers were being diagnosed that would otherwise never have become evident clinically^{4–6}. No statistics are available on the frequency or timing of clinical examinations of the thyroid in children, nor on the percentage of tumours that were picked up by screening. But the magnitude of the increase is probably too great to be due to increased screening of the population. Screening may cause at the very most a transient 10-fold increase in the frequency of thyroid cancer⁶; but the increase in thyroid cancer in the Ukraine was considerably greater than that in some areas.

In conclusion, the pattern of thyroid cancer in relation to thyroid dose from ^{131}I suggests that the increase in thyroid cancer in childhood reported in the Ukraine is likely to be a direct consequence of the accident at Chernobyl.

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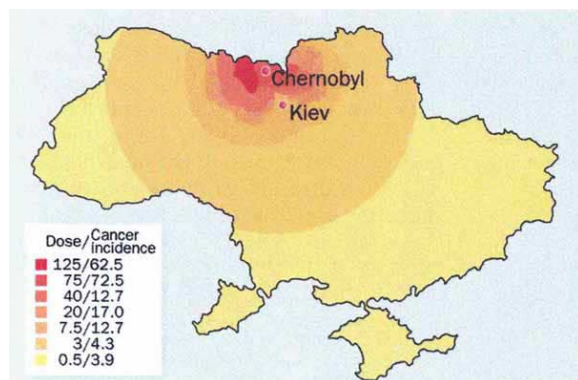
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Nailing the lophophorates

SIR — Henry Gee¹ is right to remind us that it may be premature to rely on a single molecule when the apple-cart of metazoan phylogeny is threatened with an upset. Via a series of deliciously coded nuances he defends the long-cherished opinion that the lophophorates (the bivalved brachiopods, colonial ectoprocts or bryozoans, and phoronid worms) belong within the deuterostomes (which undoubtedly include the pterobranchs), rather than the protostomes, as an analysis of 18S ribosomal DNA now indicates².

Nevertheless, this latter view is based on more than a single molecule. Consider the setae of brachiopods, chitinous



Map of the Ukraine showing the distribution of doses to the thyroid and the corresponding annual incidence of thyroid cancer per million (1990–92) for children who were 0–18 years old at the time of the Chernobyl accident. The key shows the median doses (in cGy) to the thyroid from ^{131}I corresponding to the annual incidence rates (per million). Respective numbers of thyroid cancers were 6, 5, 4, 15, 53, 46 and 98. The basis for the dose estimates is given in ref. 1.

INCIDENCE RATE OF THYROID CANCER (PER MILLION) IN CHILDREN AGED 0–14, 1986–93

	1986	1987	1988	1989	1990	1991	1992	1993
Incidence								
rate (no.)	0.72(8)	0.63(7)	0.72(8)	0.99(11)	2.35(26)	2.00(22)	4.24(48)*	3.70(42)*

*Includes one child born after 1986.

bristles that arise along the mantle margin of each valve. Their ultrastructure is identical to the chaetae of annelid polychaetes³, animals which at first glance look very different with their metameric segmentation and chaetae arising in bundles from the parapodia. Zoologists adhering to the deuterostome camp are content to accept the shared ultrastructure as yet another example of convergence. If, however, brachiopods and related lophophorates are indeed protostomes, then this similarity may become much more significant. Nevertheless, with only crown groups available, the argument of whether setae and chaetae are convergent or share a common descent is difficult to resolve.

The fossil record, however, suggests a possible way forwards. What appear to be equivalents to polychaete chaetae occur as the sclerites of the Burgess Shale animal *Wiwaxia*⁴, although overall this Cambrian worm differs in several important respects from polychaetes. Significant similarities in sclerite arrangement exist, however, between *wiwaxiids* and a slightly older group, known as the *halkieriids*. This latter group, however, has the peculiarity of possessing a prominent shell at either end of its slug-like body⁵. These shells have what may be more than a passing similarity to the valves of brachiopods. Imagine a juvenile *halkieriid*, that, rather than having its shells back to back early in its development, swung one valve beneath the other. This is not implausible given that a similar rotation of the shell rudiments as occurs in the

embryology of the living brachiopod *Crania*⁶. If we accept that the sclerites of *halkieriids* and *wiwaxiids* are equivalent⁵, but the latter have the ultrastructure of chaetae⁴, then a subsequent transformation of the sclerites that surround each shell to the setae of brachiopods becomes an intriguing hypothesis.

Suppose further evidence, including molecular data (Bernard Cohen, personal communication), places the lophophorates firmly in the protostomes. It certainly now seems to be very difficult to homologize the lophophores of the lophophorates and deuterostome pterobranchs. Their similarity now becomes convergent. Should we accept the recent proposal² that the tentacular lophophore was a primitive protostome feature and was lost in the annelids and molluscs? This hypothesis may be difficult to reconcile with the widely accepted derivation of molluscs from a turbellarian ancestor, especially if the *halkieriids* represent an intermediate condition⁵. In this alternative scheme, the lophophore is a relatively late acquisition first appearing in the brachiopods, which themselves may derive from either a *halkieriid* worm or near relative.

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Crust formation in the Lewisian

SIR — Burton *et al.*¹ have presented U–Pb and Sm–Nd mineral regression ages on rocks from the Lewisian complex at Gruinard Bay, which apparently require that “the history of crustal development in the Lewisian complex will have to be radically rethought”². They¹ consider that their data provide information about “... how the earliest continental crust may have formed”, despite being 600 Myr younger than the earliest substantial continental crust³. Furthermore, previous geochronology on mafic–ultramafic complexes (~2,900 Myr, $\epsilon_{\text{Nd}} = +2$), which could also be a potential source⁴, has been ignored.

A Pb–Pb age for the northern-region granodiorites⁵, considered to be spurious⁶, has been used (Fig. 2b of ref. 1), despite the observation that this suite does not fit the Nd evolution model (Fig. 2a of ref. 1). The latter point is explained partly on the basis of “... unusually low...” $f_{\text{Sm}/\text{Nd}}$ values, but these are indistinguishable from the Scourie gneisses (–0.507 versus –0.486; ref. 5). Trondjemite mineral regression data¹ are also assumed to be magmatic protolith ages because they fall

on the Pb and Nd isotope evolution trends for the proposed tonalite, trondjemite and granodiorite (TTG) precursor, whereas previously published whole-rock ages of 2,800 to 3,000 Myr (Sm–Nd^{4,7} and Pb–Pb⁶) are “... considered simply to reflect the age of the mantle source”¹. How can the trondjemite whole-rock age reflect the age of a source through two magmatic events (derivation of TTG precursor followed by trondjemite production)? If the whole-rock ages reflect only the age of the TTG precursor, why are these not 3,310 Myr?

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BURTON *ET AL.* REPLY — The comments of Fowler *et al.* give us no cause to change our views, for the reasons outlined in our original manuscript¹.

Fowler *et al.* suggest that mafic–ultra-

mafic complexes in the high-grade part of this terrain, to which they assign an age of ~2,900 Myr, may also be a potential source of the tonalite, trondjemite and granodiorite rock types. In fact, five mafic–ultramafic complexes have been dated, and these give ^{147}Sm – ^{144}Nd ages ranging from 2,600 to 2,900 Myr and ϵ_{Nd} values around +2 (refs 4, 8). Clearly, those that give ages around 2,600 to 2,700 Myr cannot have been a source for the Scourian tonalites ($\epsilon_{\text{Nd}} = -2$), as they are contemporaneous, and yet show a significant difference in ϵ_{Nd} at the time of regional metamorphism recorded by the tonalites. It might be claimed that the oldest of these bodies could be a potential source, but the implied evolution from mafic–ultramafics at 2,900 Myr to regional metamorphism at 2,600 Myr requires a $^{147}\text{Sm}/^{144}\text{Nd}$ ratio of 0.0834, unreasonably low for the average continental crust. Although a few (4 of 17) of the Scourie tonalites do indeed possess such low $^{147}\text{Sm}/^{144}\text{Nd}$ ratios, these result from the fractionation of Sm/Nd during regional granulite metamorphism⁷, which occurred after their formation. Moreover, as noted previously⁸, the data suggest that the older ages of around 2,900 Myr result from contamination and mixing with the pre-existing tonalites, in which case any isotopic correlation will have no time significance.

With regard to the northern-region granodiorite data, neither the Pb–Pb nor the Sm/Nd ages were used to construct the best-fit lines shown, but were included as they are discussed in the text. We are sorry that this was not made clear in our paper. The Pb–Pb age for these rocks proposed by Whitehouse⁵ was later considered to be spurious on the grounds that some of these samples had experienced extreme uranium depletion⁶. The same samples have very low Sm/Nd ratios, which suggests that they may also have undergone Sm/Nd fractionation similar to that experienced by the Scourian tonalites. The fact that these samples show similar Sm/Nd ratios to those that have experienced granulite metamorphism clearly illustrates this point. If the authors wish to place some significance on the derived age estimate (note that the data do not define an isochron; MSWD = 8.3), then they are guilty of exactly what they have criticized others for^{7,9} — that is,

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regression of samples that have experienced a different geological history to obtain a supposedly meaningful age.

The trondhjemite mineral data are taken to define an age close to the time of emplacement for the reasons given in the original paper¹. However, the whole-rock Sm–Nd and Pb–Pb ages of 2,800 and 3,000 Myr are considered to represent the age of ‘their source’, not of ‘the mantle source’. The latter phrase was not present in our final submitted manuscript, but appeared as an editorial change which we unfortunately failed to notice on the proofs.

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Energy partitions

SIR — John Maddox (*Nature* 374, 11; 1995) discusses results by Y. Du *et al.* (*Phys. Rev. Lett.* 74, 1268–1271; 1995) of a one-dimensional array of particles which undergo inelastic collisions: inelasticity implies a loss of energy, and thus energy is fed at a bounding wall to maintain steady state. Energy is not equipartitioned: it decays as one moves away from that wall.

Maddox calls the situation “tantalizing”; he believes that the result may be an artefact of the one-dimensional assumption: “So where is the snag? . . . It will be interesting to learn what the two-dimensional simulations . . . will show. A similar result would indeed be subversive. That outcome seems improbable . . .”

That outcome has been found by people who were looking at large-size particles such as found in hoppers, chutes and the like. But we first discuss a simple problem with an ideal gas, where the same result is obtained.

Consider a long tube containing an ideal gas, hotter than the surrounding ambient, so that energy is lost by heat transfer. The latter is equivalent to collision inelasticity in that it allows energy to be lost. Steady state is maintained by supplying heat at one wall. Temperature will decay as one moves away from the heated wall, and energy is not equipartitioned. All it takes is: a mechanism for loss of energy; and a steady supply of energy at one position to balance the energy loss.

J. T. Jenkins and S. B. Savage (*J. Fluid Mech.* 130, 187–202; 1983) and others have analysed the statistical mechanics of a granular material with inelastic collisions. Vigorously shake a bottle of

pills: the pills have a significant kinetic energy of oscillation, which may be kept constant if the shaking mechanical energy input exactly balances the energy loss due to inelasticity. That collisions are inelastic is shown by the fact that, as soon as shaking ceases, it will take very little time for all the pills to set down on the bottom and have a zero “temperature”.

Application of the Jenkins–Savage theory to some simple flow of granular materials quickly shows that fluctuation energy is not equipartitioned. Michael Faraday (*Proc. R. Soc. A* 121, 299–340; 1831) reported on the flow of a mound of particles sitting on a vertically vibrated plane. The same experiment was done again by Savage (*J. Fluid Mech.* 194, 457–478; 1988), who has correctly interpreted the results in terms of an inelastic collision flow theory.

There is nothing surprising about the Du *et al.* results. What is surprising is that, in rarefied gases, collisions are so precisely elastic that equipartition of energy occurs. What Maddox calls a “deliciously schooltextbook notion”, the coefficient of restitution, has to be exactly unity for equipartition to occur. Should it be less than unity, no matter by how small an amount, equipartition would not occur except in very special cases. Maddox does not need to wait for the “implicitly promised” two-dimensional simulations. Energy will not be equally partitioned.

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Complementarity and uncertainty

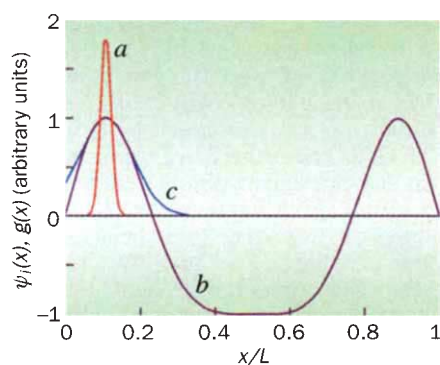
SIR — Storey *et al.*¹ claim to have shown that, in any scheme for detecting the way of a particle through a Young’s double-slit interferometer, the interference pattern is destroyed because the detector transfers momentum to the particle. The authors then conclude that the principle of complementarity is simply a consequence of Heisenberg’s uncertainty relation and not a more fundamental concept. We disagree. The principle of complementarity is much deeper than the uncertainty relation, although it is frequently enforced by $\delta x \delta p \geq \hbar/2$. We show here (1) that the general arguments by Storey *et al.* cannot be right in the face of a recent experiment; and (2) that their specific criticism of our analysis of the quantum-optical which-way detectors is invalid because the calculation is incomplete. The complete detailed calculation² confirms our original statement³ that it is the presence of which-way detectors, not

Heisenberg’s uncertainty relation, that enforces complementarity.

A which-of-two-ways-type experiment based on photons scattered from two atoms had been proposed by Drühl and M. O. S.⁴ and realized by Eichmann *et al.*⁵. In this experiment, photons emitted from a source reach a screen after being scattered off two $^{198}\text{Hg}^+$ ions held firmly at rest in a linear Paul trap. This resonant scattering occurs in two steps. First, the incoming photon is absorbed and one of the two ions is excited from its $6s^2S_{1/2}$ ground state to the $6p^2P_{3/2}$ state; then a photon is emitted and this ion returns to the ground state. Both the ground state and the excited state have two Zeeman sublevels with $m_J = +1/2$ and $m_J = -1/2$, which is the key element in the which-way experiment. The incoming photons are linearly polarized. Therefore, if one observes fluorescence photons with the same linear polarization, the final state of the scattering atom is identical with the initial one. In this case, which-way information is not available and an interference pattern is observed. By contrast, if one observes fluorescence photons that are circularly polarized, then the scattering atom ends up in a different Zeeman level from the one it was in initially. Consequently, which-way information is available in this case and no interference pattern can be observed.

The disappearance of the fringes cannot be blamed on a change in the external motion of the photon — as Storey *et al.* would conclude — because the observation of the interference for linear polarization definitively excludes the excuse of a substantial momentum kick. We thus have here a clear experimental counter-example to the notion that the enforcement of Bohr’s principle of complementarity in two-slit experiments is always a consequence of Heisenberg’s uncertainty relation.

Storey *et al.*¹ present their argument in a longer paper⁶ to which we have already replied². In Fig. 1 of ref. 6, Storey *et al.* incorrectly place the which-way detectors between the double slit and the screen. The correct location is depicted in Fig. 3 of ref. 3, where the atoms traverse the which-way detectors before reaching the slits. At this early stage, the precision with which the position has to be determined must only suffice to distinguish the upper partial beam from the lower one. The avoidance of a position measurement is a decisive advantage of our scheme³. Storey *et al.* correctly find that the final centre-of-mass wave function $\Psi_f(x) = \Psi_i(x) g(x)$ is the product of the initial one, $\Psi_i(x)$, and a function $g(x)$ that is determined by the geometry of the resonator. In the figure we show the $g(x)$ function used by Storey *et al.* and a typical initial wavefunction $\Psi_i(x)$. Note that $\Psi_i(x)$ is well localized, and therefore $g(x)$ matters only where $\Psi_i(x)$ is



Initial wavefunction $\psi_i(x)$ (line a) and the function $g(x)$ (line b) are shown as a function of x (in units of the cavity extension L). Near its left peak, $g(x)$ is very well approximated by a simple Gaussian (line c).

nonzero. There, $g(x)$ is practically constant, so that $\psi_f(x)$ differs only very slightly from $\psi_i(x)$. The extent to which they are the same is measured by their quantum mechanical overlap. For the parameters used in the figure, this overlap is short of unity by less than 2 parts in 10^4 . In other words, if nothing else were happening, only 2 out of 10,000 atoms would go astray — because of the recoil effects of Storey *et al.* — and not contribute to the interference pattern in the way they should. This tiny reduction of the fringe contrast cannot be held responsible for the total loss of the interference pattern.

Storey *et al.* did not finish their calculation because they did not compute the loss of fringe contrast that originates in the transition from $\psi_i(x)$ to $\psi_f(x)$, but focused instead on the Fourier transform of $g(x)$, depicted in Fig. 1 of ref. 1. Indeed, if one replaces $g(x)$ by the smooth function that is dashed in our figure, then $\psi_f(x)$ is essentially unaltered. The structure in the Fourier transform, to which Storey *et al.* erroneously attribute so much physical significance, disappears, however.

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STOREY ET AL. REPLY — We asked whether it is possible to destroy an interference pattern without transferring momentum¹. In interference experiments with a screen in the far field, the pattern on the screen is the distribution of outgoing momentum. As momentum is conserved in every interaction in quantum mechanics, any process that changes one pattern into another must involve a momentum transfer.

From this viewpoint, passing particles through a narrow slit involves a large

momentum transfer, that required to convert a plane wave of well-defined (usually zero) transverse momentum to a cylindrical wave with a range of transverse momenta. A similar large momentum transfer is involved in passage through two slits.

According to the principle of complementarity, the introduction of a path detector into a double-slit interferometer must change an existing interference pattern into a featureless diffraction pattern. In our paper, we showed that the minimum additional amount of momentum that must be transferred by a path detector to effect this change in the pattern is related to the slit separation d through the uncertainty principle ($\Delta p \geq \hbar/d$). This momentum transfer can be regarded as the physical mechanism that enforces the principle of complementarity in this case.

Englert *et al.* cite as a counter-example the recent experiment performed by Eichmann *et al.*⁵, who observe the distribution of light scattered by two $^{198}\text{Hg}^+$ ions held at rest in a Paul trap. The result for π -polarized light exhibits partial interference whereas that for σ -polarized light exhibits no interference. It is not valid to claim, as Englert *et al.* do, that “the observation of interference for linear polarization definitely excludes the excuse of a substantial momentum kick”, as the formation of any interference pattern involves substantial kicks. In this experiment the two ions play the role of the double slit, and must transfer a range of momenta greater than $\hbar/d$ to the photons in order for even the first fringe of the interference pattern to be visible. Unlike the situation considered in our paper, the ions also act as the path detector, and it is not possible to separate out the momentum transfer involved in the detection process alone.

Although large momentum transfers occur for both light polarizations, the distribution of transferred momentum differs between the two cases: the π -polarized light exhibits interference, whereas the σ -polarized light does not. This is consistent with the principle of complementarity, because a σ -polarized photon leaves a record of its path in the internal states of the ions. Eichmann *et al.*, however, give an alternative explanation which reveals the physical mechanism enforcing this complementarity. A calculation of the fluorescence spectrum from a single atom with the level structure of $^{198}\text{Hg}^+$ (ref. 7) shows that only the π -polarized scattered light is coherent, whereas the σ -polarized components are incoherent. A definite phase relationship exists between the π -polarized light scattered from the two ions, but for σ -polarized light, this relative phase is random. Interference is thus seen only for π -polarized light.

In analysing the destruction of interference in a two-slit configuration, we

should consider phase shifts or amplitude changes between the slits introduced by the physics of the detection process. These can alter the interference pattern in a random way so that the probabilistic average yields a featureless pattern⁸. The family of functions $O_\xi(x)$ we introduced¹ extend across both slits and precisely quantify the change to the system when the detector is found to be in state ξ . As the input wavefunction $\psi_i(x)$ passes through the system it is successively multiplied by the appropriate $O_\xi(x)$ and by the transmission functions of the various system elements so the order of the slits and path detector cannot matter. For narrow slits, the values of $O_\xi(x)$ may be changed arbitrarily away from the slit locations x_1 and x_2 . What our general arguments have shown is that no matter how the value of $O_\xi(x_1)$ is joined to $O_\xi(x_2)$, the Fourier transform for some ξ will contain components with enough momentum to reduce the visibility by the measured amount.

Englert *et al.* erroneously select a single member of this family (which they call $g(x)$) and consider its effects as extending over only a single slit. By neglecting the index ξ , the randomness associated with the detection process is lost^{3,8}, and by assuming that $\psi_i(x)$ is a single-peaked function (rather than a two-peaked function, with a peak at each of the two slits) they have effectively assumed that which-path information is already available before the interaction with the path detector.

Having made this oversight, it is not surprising that the overlap Englert *et al.* calculate between $\psi_i(x)$ and $\psi_f(x)$ is so close to unity. In effect, they are evaluating the change that a momentum transfer of the order of $\hbar/d$ makes to a single-slit diffraction pattern which can be made arbitrarily small by reducing the slit width. However, such momentum transfers are sufficient (and necessary) to wash out the interference pattern associated with a double-slit with slit separation d .

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For want of an argument

Michael Dummett

Sophie's World. By Jostein Gaarder. Farrar, Straus and Giroux/Phoenix House: 1994/1995. Pp. 403. \$19, £16.99.

A BOOK aiming to teach 15-year-olds something about philosophy, and yet be attractive to them to read, certainly evokes admiration for the courage of its author; that is no doubt a large component of the acclaim with which *Sophie's World* has been received. I should not think of embarking on such a project myself; I know hardly anyone else who would do so. My attitude to the author is therefore like that to one who, before Hillary and Tenzing, attempted to climb Mount Everest and failed. In criticizing the book, I am not purporting to know how to do better than Gaarder, merely regretfully recording that he did not reach the summit.

The book is a philosophy course, printed in one typeface, set within a fantasy, printed in another. The fantasy is at first muted: a young girl receives envelopes, delivered by hand, containing successive chapters of a history of philosophy; who has written them, and why he has chosen her as sole recipient instead of publishing a book as Gaarder has done, remain at first mysterious. But attention is focused in the earlier part of the book on the chapters on philosophy addressed to Sophie. These presumably embody the point of the book: the fantasy is only the sugar-coating to make it palatable.

I have long believed that we are wise, in England, to reserve the study of philosophy to university students, and not to teach it in school, as is done in many European countries; indeed, that Oxford is wise not to allow it to be studied by undergraduates save in conjunction with some other subject. One reason is that while it demands a high degree of reasoning ability, though not so high a degree as mathematics, it also demands what mathematics does not at all, a high degree of intellectual maturity. The other main reason is that its study at school is likely to be by means of textbooks; and a textbook in philosophy is antithetical to the entire spirit of the subject. Now Gaarder has in effect written a textbook for 15-year-olds. My guess would be that the best way of introducing adolescents to philosophy would be to ignore the history of the subject and try instead to acquaint them with the problems philosophers tackle, making sense of those problems, explaining their difficulty, sketching some solutions and

the arguments for and against them, but arriving at no conclusions. This could be done in only a superficial way; but it might convey a feeling for and interest in the subject and would certainly stimulate thought about the problems — half-baked thought, no doubt, but that would not matter.

Gaarder has not adopted this strategy at all: instead, he takes his imaginary pupil through a lightning course in the history



of Western philosophy (the philosophy of India and of Islam are left unmentioned). The resulting textbook indeed conveys some of the leading thoughts of some of the leading philosophers — some much more successfully than others; but it lacks the chief defect of textbooks of philosophy for the young and thereby incurs another. The chapters do not end with enumerations of the principal arguments for and against the views of the philosopher surveyed; the book thus escapes the most deadening effect of a philosophy textbook. The result, however, is that it almost completely fails to convey the centrality of argument to philosophy, which is nearly as great as the centrality of proof to mathematics. It is true that a philosopher who merely makes assertions without offering grounds for them may still make an important contribution; but his assertions are open to attack by argument and stand in need of defence by argument.

Sophie learns some important features of philosophy. She learns that a philosopher must retain, or regain, a sense of wonder and that he must not take for

granted anything that he knows or believes, but must ask how he knows or why he believes it and how things would be if it were not so. But she does not learn the salience of argument in the subject and thereby fails to grasp that, despite the many absurdities philosophers have committed, philosophy is a supremely rational mode of enquiry. In failing to convey this to her, Gaarder has left her ignorant of one of the prime characteristics of philosophy.

The chapters of the textbook are devoted, in succession, to: ancient myth; the pre-Socratics; Democritus; classical fatalism; Socrates; Plato; Aristotle; Hellenistic philosophy; Judaism and Christianity; St Augustine and St Thomas Aquinas; the Renaissance (Ficino to Newton); materialism; Descartes; Spinoza; Locke; Hume; Berkeley; the Enlightenment; Kant; Hegel; Kierkegaard; Marx; Darwin; Freud; and Jean-Paul Sartre. In the course of these chapters, other philosophers and schools are occasionally mentioned glancingly. One might complain that philosophy since Hegel has been treated so churlishly. Analytical philosophy is mentioned in one sentence: "Another [current] is the so-called *analytical philosophy* or *logical empiricism*, with roots reaching back to Hume and British empiricism, and even to the logic of Aristotle."

Here the revolution in logic initiated by Frege, which underlies analytical philosophy, is obliterated; neither Frege nor Wittgenstein (who detested Hume) is so much as mentioned in the book. But none of this matters unduly. Sophie has been given a framework, even if it is missing several bars, and if she becomes interested in philosophy as a result, she will, or should, soon learn better.

What does matter is that one of the salient ideas of which most philosophers have made use goes virtually unmentioned: the distinction between the necessary and the contingent. This is not indeed uncontentious: whether the distinction is sharp or blurred, even whether it is useful or useless, and how many types of necessity there are, remain live issues. But it has played a major role in philosophical thought; it is a grave omission to leave it out. This may in part be due to the exclusion of Leibniz from the roll of philosophers discussed. It inescapably plays a part in the account of Kant, but not an explicit one or one linked to the views of other philosophers on the distinction.

As the book proceeds, the fantastic strain becomes ever more pronounced. Perhaps this is intended to retain a grasp on the attention of the adolescent readers; but I think it is a mistake. It is likely, rather, to capture all their attention, so

exciting does the fantasy become; they will probably only skim the later lessons. It eventually turns out that both Sophie and her philosophy teacher are merely characters in a book written by a major for his daughter Hilde; but they contrive to escape from the book into a shadow world inhabited by other fictional characters such as Lewis Carroll's Alice. This of course is nonsense; although things may happen in a book of exactly the same kind as those that happen in real life, we could not discover that we were only characters in a book. The idea is very well in a work of fantasy but grossly out of place in a book meant to instruct in philosophy. Possibly it is intended to stimulate the young readers into thinking out just why it is nonsense, but, if so, I regard it as very ill judged: all but the coolest thinkers among them will be so entranced by the mystery and adventure that they will not stop to think about this at all.

The British edition of the book has been translated into American, not English, English. For the most part this is only occasionally obtrusive; but we are sometimes brought up abruptly by hideous constructions such as "They tried to not even look at it". British publishers used to take care that British editions of their books — even of books written by Americans and first published in the United States — came out in the English used in their country. Now they plead that they cannot make a profit unless their books sell in the United States and that therefore they must be written in the American dialect. If their plea were sound, it would surely follow that no books would be published in Italian or in Norwegian, and hence that this book could not exist. Perhaps this is a further paradox for its adolescent readers to unravel. □

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Back to the future

John Postgate

Haldane's *Daedalus* Revisited. Edited by Krishna R. Dronamraju. Oxford University Press: 1995. Pp. 147. £19.99.

THE 1920s were times of glorious cultural and intellectual turbulence for young, forward-looking Westerners. The First World War, with its attendant problems of conscience, was receding into history; communism had triumphed in Russia, an event confidently expected to transform that society into a shining example of equality, liberty and fraternity. The Victorian ethos was in retreat at all levels: religion and *laissez-faire* capitalism were *démodé*; positivism was undermining

traditional philosophy and metaphysics; postwar youth was challenging accepted sexual and behavioural mores; surrealists, dadaists and the cinema were bringing about new and sometimes shocking changes in the visual arts; and, for the first time in several centuries, a truly original form of music had arisen — jazz. At a more material level, daily lives were being transformed by the fall-out of scientific



J. B. S. Haldane: armchair prophet.

advance: radio, telephones, motor cars and air travel. Despite hiccups such as hyperinflation in Germany, the General Strike of 1926 in the United Kingdom and the rise of a fascist dictator in Italy, and despite the doubts, even antagonisms, of romantics such as G. K. Chesterton and E. M. Forster, the mood was optimistic: enlightened politics combined with progress in science, medicine and technology bade fair to solve all the more pressing problems of society.

Science was still part of an educated person's repertoire. Admittedly it was no longer universal as it had been in the late nineteenth century, when Darwin's *Origin of Species* could sell out on its publication day, but books about, or based on, science were still widely read, and H. G. Wells, in particular, had shown that technical and scientific themes could be both exciting and predictive. So when, in 1924, Haldane published *Daedalus*, a slim volume of his own prophecies, he could be sure of a wide audience. It was very much of its time. Brash confident and didactic, *Daedalus* set out to shock, to make its readers re-think their beliefs, risking error, overstatement and semantic carelessness (technology and science were lumped together). The book caused a

sensation. Among several controversial features, it dismissed religion and nationalism almost casually, it accepted socialism, it separated sex from procreation, prophesying widespread "ectogenesis", by which he meant the culture of eugenically selected babies *in vitro* (an idea that Aldous Huxley developed tellingly in his *Brave New World*). He presented his more startling predictions as an imaginary essay by a rather stupid undergraduate of "150 years hence". As we approach the halfway mark, *Daedalus* still makes thought-provoking reading; Haldane's hits and misses were about evenly balanced.

The reprint here is preceded by Joshua Lederberg's foreword (which draws some morals for today from *Daedalus*) and introduced by Dronamraju (who knew Haldane well during his final seven years in India) with a concise account of Haldane's life and thinking. Then come reflections by some distinguished intellectuals of today, presumably prompted by *Daedalus*. I write 'presumably' because the first, by Max Perutz, has no obvious relevance to *Daedalus* at all, being a couple of pages of personal recollections of Haldane prepared for an earlier centenary celebration. More relevant, and in keeping with *Daedalus* in blithely going over the top, is Freeman Dyson's chapter. He inveighs against science, equating, as did Haldane, its intellectual structure with the consequences of its applications, choosing especially those that are unpleasant, war-like and socially disruptive. It amounts to aggrieved and muddled Greenpeace fodder; yet his conclusion, that "ethical progress is the only cure for the damage done by scientific progress" would have been unexceptionable had he replaced the word 'scientific' by 'technological'.

Yaron Ezrahi then considers the philosophical status of Haldane's thinking. Like Bernal, Monod and many scientists today, Haldane believed that scientific rationality could be a model for human affairs. Ezrahi finds this proposition incompatible with democracy, since most human affairs involve action and decisions based on data that are neither consensual nor complete. Moreover, on the one historical occasion when it was tried, he points out, it led to Stalinism. *Touché* — but in principle I could not disagree more. However, his view that science has today lost its cultural authority, as Haldane feared it would, is certainly correct.

Ernst Mayr contents himself with an overview of *Daedalus*, including some common sense on the emotive topic of eugenics, and a consideration of how

New in paper back

Was Einstein Right? Putting General Relativity to the Test

by Clifford M. Will. Oxford University Press, £8.99. Now in its second edition, this award-winning exposition of how Einstein's theory of general relativity holds up to scientific scrutiny has been updated to cover the most recent experimental findings, including the story of the rise and fall of the 'fifth force'. The author, a professor of physics at Washington University in St Louis, combines the personal with the theoretical. "What I find truly amazing," he writes, "is that this theory of general relativity, invented almost out of pure thought, guided only by the principle of equivalence and by Einstein's imagination, not by a need to account for experimental data, turned out in the end to be so right."

The Undivided Universe: An Ontological Interpretation of Quantum Theory

by D. Bohm and B. J. Hilley. Routledge, £12.99. David Bohm, who died in 1992, spent much of his life developing an idiosyncratic interpretation of quantum mechanics. His idea is that the electron is a point particle as in classical mechanics, but that its trajectory is influenced by what he called the "quantum potential", a new type of non-classical physical field derived from the wave-function. The book is intended to bring this idea up to date. Using mathematics, the authors describe how many of the statistically predicted phenomena of quantum mechanics can be explained 'ontologically' in terms of an ensemble of individually well-defined causal motions. For a review, see *Nature* 366, 420 (1993).

The Essence of Chaos by Edward Lorenz. UCL Press, £10.95. In this introduction to chaos theory, the author gives an insider's view of the formative years of the field, including the true source of the notorious 'butterfly effect'. The idea of a butterfly flapping its wings in Brazil and setting off a tornado in Texas was apparently anticipated in George R. Stewart's novel *Storm*, in which a meteorologist recalls his professor's remark that a man sneezing in China may set people shovelling snow in New York.

Total Eclipses of the Sun by J. B. Zirker. Princeton University Press, \$12.95, £10.95. A clearly written, up-to-date guide for the advanced amateur or professional astronomer that explains what causes total eclipses and how they can be used in experiments to examine such phenomena as the dust between the planets and general relativity. This expanded edition includes a chapter on the "great Hawaiian eclipse" of July 1991.

Haldane might have viewed the state of science today. Eugenics also forms the background of Elof Axel Carlson's comparison of the outlooks of Haldane and geneticist H. J. Muller, who were both convinced eugenicists as well as Marxists for most of their lives.

A scholarly chapter by David Weatherall combines a detailed survey of *Daedalus* with a wise and witty consideration of how far medical science has matched it today, and how one might now revise its predictions. Thus, the spartan requirements of preventative medicine would cause transient psychiatric problems that would "gradually pass as generations are born which have never known the pleasures of cream teas, good claret, and a decent

cigar". That came from the heart! The penultimate chapter is by Haldane's nephew Avrion Mitchison. Like Perutz's, it has but a tenuous link with *Daedalus*, being an authoritative review of the prospects for human immunology. Although written in an easy style, it assumes a fair amount of background knowledge in the subject. Finally, Dronamraju, in a respectful postscript, comments on Haldane's ambivalent views on religion. A disorganized, repetitious but stimulating compilation of hits and misses — not unlike *Daedalus*, really. □

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The briefest of histories of time

Graham Farmelo

Stephen Hawking for Beginners. By J. P. McEvoy and Oscar Zarate. Icon: 1995. Pp. 175. £7.99 (pbk)

In this age of the potent image and the snappy sound-bite, it is becoming harder by the year to present anything that requires an attention span much longer than a television commercial. In this climate, it was inevitable that someone would have the idea of presenting great contemporary icons, themes and ideas to beginners in the form of 150-page strip cartoons.

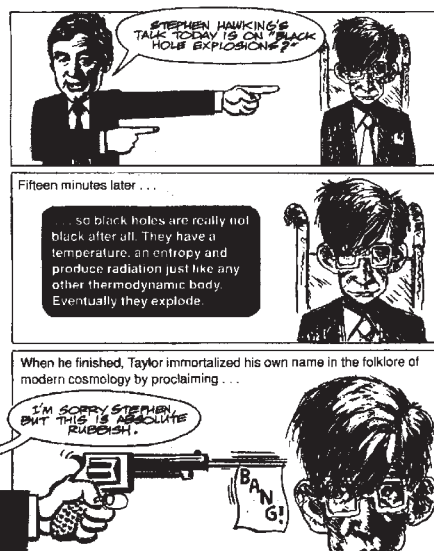
Icon Books is among the leaders in this field. Over the past few years it has published dozens of beginners' guides, many of them about science. Their contents are an engaging postmodernist potpourri of line-drawings, photographs and diagrams, annotated with lively and well informed commentaries. Although the guides are not for the straight-faced and intellectually fastidious, they are justly respected for their accuracy and integrity. (One hopes this reputation will survive the forthcoming publication of an introduction to astrology.)

The latest scientist to be Iconized is Stephen Hawking, who we are told cooperated with the guide's author, the science journalist J. P. McEvoy. Many readers who gave up the unequal struggle with *A Brief History of Time* will welcome the fresh approach to that material taken in this accessible, if somewhat hagiographic, guide. Many will obtain their money's worth just from the fine section on black-hole radiation, which is marred only by its spacetime diagrams, which will be comprehensible only to *aficionados*.

There is, however, no excuse for the number of inaccuracies, misstatements and sloppily introduced ideas in the rest of the book, such as an unforgivably con-

fused passage on the units of mass and weight and an introduction to special relativity that does not even allude to inertial frames.) Do we really need five pages on Hawking's audience with the Pope when the wave-particle duality and the anthropic principle are treated with a brevity that defeats the point of their inclusion?

After his somewhat optimistic implication that Cygnus X-1 is a black hole and



From Stephen Hawking for Beginners

February 1974 at the Rutherford-Appleton Laboratory, Oxford.

an absurdly overblown account of the 1992 'cosmic ripples' observations, McEvoy struggles to explain why his hero has not been awarded a Nobel prize. There will be also no prizes for this book, I fear, at least not until it has been given an editorial spring-cleaning. □

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Life's forms

William R. Taylor and
N. Michael Green

Structure in Protein Chemistry. By Jack Kyte. Garland: 1995. Pp. 606. \$62.

"WHAT is the secret of life?" I asked. . . .
"Protein", the bartender declared. "They
found out something about protein."

The storyteller in Kurt Vonnegut's novel *Cat's Cradle*, having posed the ultimate biological question, unfortunately did not pursue the bartender's perceptive answer. Instead, he continued to seek for Truth in L. B. Johnson's "Books of Bokonon", which, despite being a source of much wisdom, have very little to say about proteins and nothing at all about protein structure.

The encounter took place in the early 1960s and the event to which the bartender refers is, obviously, the elucidation of the first globular protein structure by X-ray crystallography. But following the narrow path from the structure of myoglobin to the answer to the ultimate question requires more than simply contemplating the fruits of the crystallographer's trade for long hours on the computer screen: the modern molecular acolyte must also master the world of atoms and forces.

When every presentation of a protein structure in today's literature is a shiny sinuous sculpture, it is all too easy to forget chemistry and simply accept the view that proteins are made of multicoloured Play Doh. This growing misconception is seldom dispelled with any conviction in any of the relevant textbooks: most of them attempt to cover the whole of biochemistry and, in the limited space accorded to protein structure, they move quickly from the structure of the 20 amino acids, through secondary structure, to the more abstract representations of protein folds. Exceptions can be found in the more specialized volumes such as *Principles of Protein Structure* by G. E. Schulz and R. H. Schirmer (Springer, 1979) and the more recent and comprehensive *Proteins: Structures and Molecular Properties* by T. E. Creighton (2nd edn, W. H. Freeman, 1983).

To this limited selection of textbooks on protein structure, Jack Kyte has contributed a volume covering the whole range of structural levels (from atomic orbitals to protein assemblies) as well as most of the physico-chemical techniques required for the purification and characterization of proteins. *Structure in Protein Chemistry* is one of a pair of large volumes aimed at the advanced undergraduate upwards and would probably be best suited to someone entering research in

the field, either at pre- or post-doctoral level. In the companion volume, *Mechanism in Protein Structure* (Garland, 1995), Kyte deals with mechanism, and so the 'structure' volume contains little or nothing about function, catalysis or allosteric effects — even the chapter on evolution is presented largely in terms of structure. It is no doubt intended that each volume should be able to stand alone, but there can't be many students who would want to study the symmetry of oligomeric proteins without making reference to allosteric interactions. Even with both volumes to hand, gaining the complete view would not be easy: surprisingly, the 'structure' volume contains no cross-references to (or even mention of) the 'mechanism' volume.

Kyte aims to take the student back to fundamentals — "to develop in the student the ability to draw her own conclusions from only the experimental results". This goal explains the size and level of detail in the work and also the sometimes pedantic avoidance of the slightly loose terms in common usage. For example, 'structure', which is often applied to the three-dimensional representation of a protein, is replaced throughout the text by 'crystallographic molecular model' (or its NMR equivalent) and, similarly, 'Bragg spacings' is used where 'resolution' would be expected. This approach extends also to the representation of detailed stereochemistry in which all the lone-pair electrons are indicated, turning a molecule even as simple as SO_2 into something more like a diagrammatic analysis of a snooker break. Undoubtedly, there are times when such exactness is required, but when applied so relentlessly, the flow of the text falters and even comprehension of some more difficult points is obscured. But if the student perseveres, he or she will arrive at a more accurate, and perhaps deeper, understanding of many of the topics.

Most modern general textbooks on proteins, such as Creighton's, use specially prepared figures that can be greatly enlivened by the use of colour, as in C.-I. Brändén and J. Tooze's *Introduction to Protein Structure* (Garland, 1991). In addition, the layout is often highly structured (with many short sections in a hierarchical order) making it easy to locate a section of interest and quickly find an answer to some specific point. Kyte's volume departs from this modern trend; the figures (apart from small organic structures) are almost all taken directly from the research literature and the sections can be long (nine pages on average). But with a comprehensive index and key words printed boldly in the text it is not too difficult to locate the relevant segment.

Despite some negative aspects (most

of which annoy rather than mislead), the volume is a valuable and unique contribution to the literature on protein structure. In emphasizing experimental studies, it presents a coherent and detailed background to many 'methods' often glossed over (or omitted) elsewhere. One of the strongest aspects of the work is the extensive lists of references after each chapter, which provide access to the research literature not available since Schulz and Schirmer's volume. This will make the book particularly useful to research students who need to understand the subject in depth (or at least give an impression of doing so). Unfortunately, some of the reference lists are now out of date, with almost no coverage of developments since 1990. For example, only one NMR-based structure is discussed and there is little on mass spectrometry. More seriously, the section on microtubule assembly is ten years behind the times and there is almost nothing on computer methods for sequence modelling and structure prediction—indeed the corresponding sections Schulz and Schirmer's 1979 textbook still provide a better account.

Should we, then, cast aside our 14 Books of Bokonon in favour of one (or two) by Kyte? We would certainly learn more about protein structure and also quite a lot of chemistry (but not, alas, the structure of ice-IX). The important question though is how close this will take us to the secret of life. Somewhere between atoms (obviously dead) and cells (obviously alive) lies the key transition. In *Chance and Necessity*, Jacob Monod tells us that proteins are purposeful (equating them with Maxwell's Demons). As this is clearly a life-like property, where better to start our search than by acquiring a full and detailed knowledge of protein chemistry and structure? □

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Biochemistry textbooks

New editions of two influential textbooks in biochemistry have just been published. The fourth edition of *Biochemistry* by Lubert Stryer (W. H. Freeman, £29.95) has been reorganized to accommodate new advances in recombinant DNA technology, protein chemistry and structural biology. There is a new chapter on protein folding and design. The second edition of *Biochemistry* by Donald Voet and Judith G. Voet (Wiley, \$76.95) also covers our new understanding of nucleic-acid and protein structure. It has an increased emphasis on human disease and makes extensive use of molecular biological techniques and molecular-graphics illustrations.

Thermodynamic observation of first-order vortex-lattice melting transition in $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$

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The lattice of magnetic flux lines that can permeate a type II superconductor, such as the high-transition-temperature copper oxide materials, melts from a solid-like state to a liquid-like state at a temperature below the superconducting transition temperature. Contrary to the predictions of mean-field theory, this phase transition in $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ is found to be first-order. The vortex liquid discontinuously expands on freezing.

THE discovery of high-transition-temperature (high- T_c) superconductors has revived the interest in study of the nature of the mixed state, where superconductivity coexists with the inhomogeneous magnetic field inside the material. Magnetic fields penetrate conventional type II superconductors in the form of a periodic array of vortices or flux lines known as the Abrikosov lattice¹. The temperature-dependent critical fields $H_{c1}(T)$ and $H_{c2}(T)$ confine the domain of the mixed state on the H - T phase diagram. Fields above H_{c2} destroy superconductivity, whereas fields below H_{c1} at the surface of a sample do not penetrate into the bulk of the material.

In high- T_c superconductors the combination of high operating temperature, short superconducting coherence length and high anisotropy enormously enhances the role of thermal fluctuations of the flux lines, resulting in a noticeable change in the nature of the mixed state. The most important effect of thermal fluctuations is the possibility of melting of the vortex lattice at temperatures well below the superconducting transition temperature^{2,3}. The resulting vortex-liquid phase may occupy a significant part of the H - T phase diagram⁴. In addition, the inevitable presence of defects and disorder in the material gives rise to pinning of the flux lines, which enriches the variety of different possible vortex states. In particular, it has been demonstrated that quenched disorder drives the vortex lattice into a vortex-glass state⁵⁻⁸. It is now believed that the freezing of the vortex liquid into a disordered vortex solid in sufficiently impure high- T_c superconductors crystals occurs via a continuous second-order phase transition^{4,6}.

On the other hand, it was suggested⁹ that the mean-field formation of the vortex lattice at H_{c2} , which occurs via a thermodynamic second-order transition, can be driven first-order by the superconducting fluctuations. One may therefore expect that in sufficiently clean crystals the vortex liquid freezes into a solid through a thermodynamic first-order phase-transition.

Additional effects may arise from the layered structure of high- T_c superconductors. In layered materials, vortex-lattice melting may occur in two separate stages: the melting of the vortex lattice into a liquid of vortex lines, and the subsequent loss of coherence between the layers—the decoupling transition¹⁰⁻¹². At moderate anisotropy the decoupling of a vortex-line liquid into a liquid of two-dimensional pancake vortices can be a first-order phase transition as well¹².

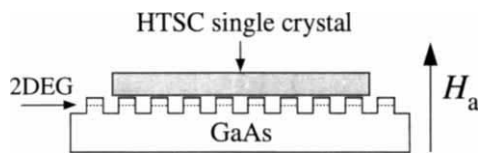
The structure of the different possible vortex states and the nature of the thermodynamic transitions between the phases has

become one of the central questions of the physics of the mixed state and has attracted much recent attention¹³⁻¹⁵. In view of the traditional mean-field prediction of a second-order phase transition¹, the possibility of a thermodynamic first-order transition of the vortex-lattice is of fundamental interest⁴. Experimentally, the main evidence for the existence of first-order melting has been drawn from the observation of sharp kinks in the resistive transition of clean $\text{YBa}_2\text{Cu}_3\text{O}_7$ (YBCO) crystals¹⁶⁻²¹. However, resistivity is not a thermodynamic property, and this kink reflects a change in vortex dynamics that does not necessarily mark a transition between distinct thermodynamic phases. At the same time, a true first-order phase transition should have clear thermodynamic features: latent heat and a discontinuous step in specific volume or density. In addition, one would expect a similar behaviour in all high- T_c superconductor materials, not only in YBCO. Recently, vortex-lattice melting in $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ (BSCCO) was inferred from disappearance of the neutron diffraction pattern at elevated temperatures¹⁵. This result implies that a vortex-liquid phase occupies a significant part of the H - T phase diagram of BSCCO; however, the order of the phase transition between the solid and the liquid phases cannot be determined. A thermodynamic indication of a possible first-order transition was recently obtained by magnetic measurements on BSCCO crystal²². Owing to limited resolution, however, the observed change in magnetization was relatively broad and not well quantified.

Here we present a very clear and direct thermodynamic observation of a first-order phase transition of the vortex lattice by use of local vortex-dynamics measurements. The local field, B , shows a discontinuous step at the transition, analogous to the discontinuous change in the specific volume on melting of a regular solid. The sensitivity of our measurement allows an accurate mapping of the melting line and the associated entropy change.

Experimental details

It was shown previously^{23,24} that the magnetic field B inside a platelet crystal is not uniform even at equilibrium magnetization. This situation is comparable to a regular solid under non-uniform pressure across the sample. As a result, the vortex-lattice melting transition occurs at a slightly different temperature or applied field at various positions inside the superconductor sample. Therefore the signal integrated over the entire sample in a standard global magnetization measurement will display a



significantly broader and smoother transition compared with the underlying physical mechanism. To probe the true width of the phase transition, local measurements with high spatial resolution are therefore required. For this purpose we have used an array of microscopic Hall sensors realized in a two-dimensional electron gas formed at a GaAs/AlGaAs interface as shown schematically in Fig. 1. A high-quality BSCCO crystal of $\sim 0.7 \times 0.3 \times 0.1 \text{ mm}^3$ ($T_c = 90 \text{ K}$) was put directly onto the surface of the probes. The two-dimensional electron-gas (2DEG) active layer resides only $\sim 1,000 \text{ \AA}$ below the surface. As a result a very accurate measurement of the local magnetic field is obtained at each of the sensor locations across the crystal.

The phase transition

A first-order phase transition is associated with a discontinuous step in density. Because each vortex carries a magnetic flux, a step in vortex density is identical to a step in the local macroscopic magnetic field. Hence, by a careful measurement of the local B_z at the surface of the sample, we can directly observe the thermodynamic discontinuous change in the vortex density at the phase transition. This discontinuity should occur as the transition line is crossed either by sweeping the applied field or by sweeping the temperature. We have carried out measurements in both regimes. Figure 2 shows the discontinuous step in B_z as the applied field is increased at a constant temperature of 80 K. The width of the transition is less than 0.4 Oe. This step occurs at the same thermodynamic value of the local B_z at various locations across the sample, but at different values of H_a owing to the non-uniform B_z profile^{23,24}.

Figure 3 shows two examples of temperature sweeps at constant fields of $H_a = 53$ and 240 Oe. The observed transition is extremely sharp. In most of our measurements the entire transition occurs between two adjacent measurements. The width of the transition is therefore less than our temperature resolution of about 1 mK. Such a discontinuous step in vortex density is a clear thermodynamic demonstration of a first-order phase transition. It is important to note that B_z in the liquid phase above the transition is higher than in the solid phase, which means that the vortex liquid is denser than the vortex solid, in contrast to the common behaviour of solids. Thus the vortex-liquid is similar to water, which expands on freezing into ice. Such behaviour is

FIG. 1 Schematic diagram of the experimental setup (not to scale). An array of ten sensors with an active area of $3 \times 3 \mu\text{m}^2$ each, is etched in a GaAs/AlGaAs heterostructure. A superconductor crystal is put directly on the surface of the probes. The two-dimensional electron-gas (2DEG) active layer resides only $\sim 1,000 \text{ \AA}$ below the surface. As a result a very accurate measurement of the local magnetic field is obtained at each of the sensor locations across the crystal.

in fact consistent with equation (1) below. On increasing the temperature a small hysteresis is observed with a slightly rounded transition that looks very similar to the reported resistive hysteresis in untwinned YBCO crystals^{18,20}. The controversial origin of the hysteresis²⁰ requires further investigation of this effect. As an example of the sensitivity of our measurement, at 240 Oe there are about 100 vortices in the area of each Hall sensor, and the measured step in B_z corresponds approximately to an addition of only 0.1 vortex to the area.

The resulting vortex-lattice melting line, $B_m(T)$, is shown in Fig. 4. The squares are points obtained by temperature sweeps, and the circles are the field sweep results. The existing theoretical derivations of the vortex-lattice melting line based on the Lindemann criterion^{25–28} predict a power-law behaviour that can be approximated by $B_m(T) = B_0(1 - T/T_c)^\alpha$, where $\alpha \leq 2$. The solid curve in Fig. 4 is a best fit to the data, which results in $\alpha = 1.55$, $B_0 = 990 \text{ G}$, and $T_c = 94.2 \text{ K}$. The power law fit describes well the behaviour in the central region; however, there are significant deviations at both high and low temperatures. On the other hand, the vortex-line liquid to vortex-pancake liquid decoupling line is expected to follow^{11,12} $B_D(T) \approx B_0(T_c - T)/T$, with $B_0 \approx \alpha_D \varepsilon^2 \phi_0^2 / (4\pi \lambda(0))^2 T_c d$. Here $\alpha_D \approx 0.1$ is a universal constant, $d = 15 \text{ \AA}$ is the interlayer spacing, $\lambda(0) \approx 2,000 \text{ \AA}$ is the penetration length, and ε is the anisotropy ratio. A fit to the experimental data (dashed curve in Fig. 4) results in a better agreement at high temperatures, with a more realistic $T_c = 90.9 \text{ K}$ and $B_0 = 400 \text{ G}$. Using the above parameters we obtain $\varepsilon^{-1} \approx 140$, which is consistent with existing estimates²⁹.

At lower temperatures, the melting line shows a rather sudden flattening, in contrast with the theoretically predicted behaviour^{11,12,25–28}. However, the most striking feature of $B_m(T)$ is the existence of a well-defined critical point at a characteristic temperature of 37.8 K and field of 380 G, beyond which no discontinuous transition is observed. Figure 5 shows the measured height of the discontinuous step at the transition, ΔB , along the melting line. We have carefully measured ΔB in the vicinity of the critical point by temperature sweeps at constant H_a , and have changed H_a by small increments. ΔB is very well resolved at fields below the critical point. However, when H_a is increased only by 1 Oe above the critical point, ΔB disappears completely. The step height ΔB increases monotonically with T_m and reaches a maximum value of about 0.4 G at $T_m \approx 83 \text{ K}$. At higher temperatures ΔB drops rapidly as T_m approaches T_c . The reason for this general form of $\Delta B(T_m)$ is not obvious.

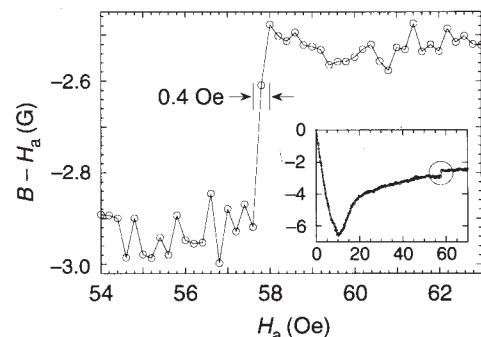


FIG. 2 Step in the local field, B_z , as the melting line is crossed by increasing the applied field at 80 K. Solid line is guide for the eye. The inset shows the entire local magnetization curve, $B - H_a$, as a function of increasing applied field H_a . The melting transition region is encircled.

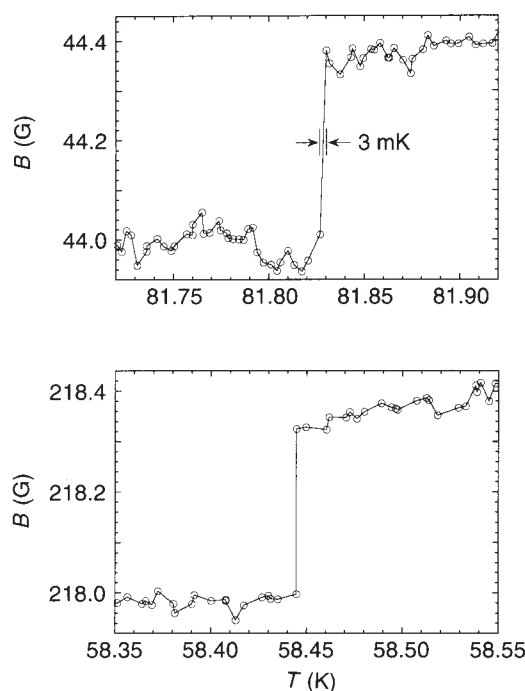


FIG. 3 Two examples of the step in local B on crossing the melting line by decreasing the temperature at constant applied fields of 53 Oe (top panel) and 240 Oe (bottom panel). Solid lines are guides for the eye. The sample was cooled very slowly, at a typical rate of 5 to 15 mK min⁻¹, while B_z and T were measured at constant time intervals.

Using the Clausius–Clapeyron relations we can calculate the entropy change at the transition and the latent heat per unit volume

$$\Delta S = -\frac{\Delta B}{4\pi} \frac{dH_m}{dT} \quad L = T_m \Delta S \quad (1)$$

The entropy change per vortex per CuO layer is therefore given by

$$\Delta s = -\frac{d\phi_0}{4\pi} \frac{\Delta B}{B_m} \frac{dH_m}{dT} \quad (2)$$

In platelet geometry the field, H , is generally not known; it is position dependent and differs from H_a . Because in our case $B_m(T) \gtrsim H_{c1}(T)$, we can approximate dH_m/dT in equations (1) and (2) by dB_m/dT , which is measured directly with high precision. The resulting Δs is shown in Fig. 6. Close to T_c the entropy change per vortex increases rapidly with temperature, which could be related to critical fluctuations. Note that ΔS per unit volume approaches zero at T_c ; however, at high temperatures B_m decreases faster than ΔB , which results in Δs increasing with temperature as determined by equation (2). Close to T_c the error bar in Δs is large and it is plausible that Δs does eventually vanish at T_c .

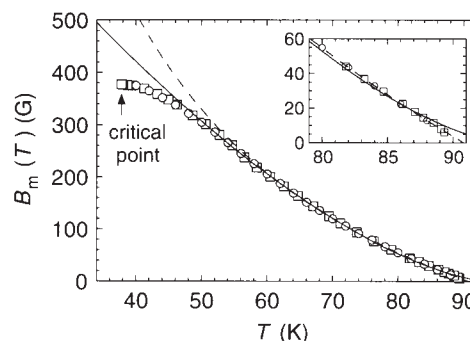
At lower temperatures, $T_m < 75$ K, the entropy change, Δs , decreases linearly with decreasing temperature and vanishes at the critical point as it should. But one would expect a continuous vanishing of ΔB as well. In contrast, ΔB seems to remain finite

at temperatures above the critical point, as shown in Fig. 5, and we could not detect any gradual disappearance of the field step. The continuous vanishing of ΔS , however, is determined by the vanishing of dB_m/dT due to the flattening of $B_m(T)$ close to the critical point (see Fig. 4), and not by the vanishing of ΔB as expected. The existence of the critical point is possibly disorder-related, in which case at lower temperatures a second-order melting line should continue from this tricritical point. This feature was observed previously²¹ in YBCO crystals by using resistive measurements. The position of the critical point was reported to shift to lower fields with increasing disorder (D. J. Bishop, personal communication).

Discussion

The latent heat and ΔB can be estimated as follows. At the melting temperature the free energies F of the solid and the liquid phases are equal and hence $\Delta F = \Delta U - T_m \Delta S = 0$. The energy required to transform a unit volume of a vortex solid into a liquid is estimated as $\Delta U \approx c_L^2 c_{66}$. Here $c_{66} \approx \phi_0 B_m / (8\pi \lambda)^2$ is the shear modulus of the vortex solid that vanishes in the liquid phase, and $c_L \approx 0.2$ is the Lindemann number. As a result, $\Delta S \approx c_L^2 c_{66} / T_m$. Making use of the estimate of the melting temperature⁴ $T_m \approx 10.8 c_L^2 c_{66} \epsilon a_0^3$, where $a_0 = (\phi_0 / B_m)^{1/2}$ is the intervortex spacing, one can evaluate³⁰ the entropy change per vortex per layer as $\Delta s \approx (0.1 d/\epsilon) (B_m / \phi_0)^{1/2}$. Accordingly, $\Delta B \approx -(0.4\pi/\epsilon) (B_m / \phi_0)^{3/2} / (dB_m/dT)$. Using the theoretical T_m and taking $\lambda(0) = 2,000$ Å, we find $\Delta B \approx 0.2$ G at low temperatures, which compares favourably with the experimental results.

FIG. 4 First-order phase-transition line in BSCCO as measured by field scans (○) and temperature scans (□). The solid line is a fit to $(1 - T/T_c)^n$ vortex-lattice melting transition behaviour; the dashed line is a fit to the $(T_c - T)/T$ decoupling transition. Inset: phase-transition line $B_m(T)$ in the vicinity of T_c .



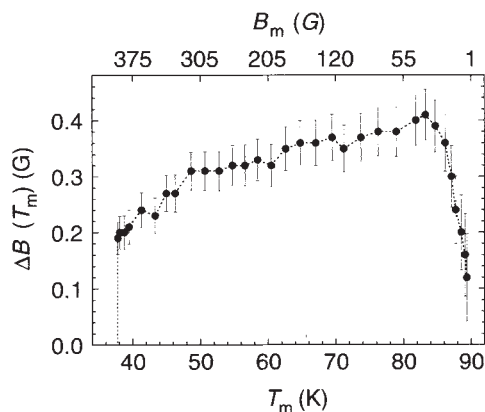


FIG. 5 The height of the field step at the melting transition as a function of the melting temperature, T_m , as derived from the temperature scans as in Fig. 3. Field scans yield very similar results. The dashed line is a guide for the eye. The field step disappears below the critical point. The corresponding approximate B_m values are shown along the top.

Note, however, that the above analysis results in $\Delta s(T) \propto (1 - T/T_c)$ close to T_c and apparently does not describe the observed increase in Δs with temperature. The resulting temperature behaviour of $\Delta B \propto (1 - T/T_c)^2$ seems to overestimate the observed rate of decrease in ΔB with temperature close to T_c .

As mentioned above, another possible origin of the observed effects is the decoupling transition. Although giving somewhat better consistency with the experimental observations, this model cannot explain the overall temperature dependence of ΔB and Δs as well. In this case the energy necessary to decouple a unit volume of the vortex-line liquid is $\Delta U \approx E_J/2d$, where $E_J \approx a_D \epsilon^2 \phi_0^2 / (4\pi\lambda)^2 d$ is the Josephson coupling energy between layers per unit area, and the decoupling temperature is $T_D \approx E_J/a_0$ (refs 4, 11). Proceeding as above we obtain straightforwardly $\Delta s \approx 1/2a_0d$, and a temperature-independent $\Delta s \approx 0.5k_B$ (ref. 12). Near T_c we find $\Delta B \approx 2\pi(T_c - T)/\phi_0d$, yielding $\Delta B \approx 0.3$ G at 80 K, and $\Delta B \approx 2\pi T_D/\phi_0d$ at low temperatures. However, the neutron diffraction data¹⁵ seem to favour the melting interpretation. The data indicate the presence of an ordered state at lower temperatures and the disappearance of long-range order at the transition. It is quite plausible, however, that the melting and decoupling transitions occur simultaneously or quite close to each other at low fields in BSCCO³¹.

In Monte-Carlo simulations of vortex-lattice melting³² $\Delta s \approx 0.3k_B$ was obtained, which is within the bulk of our data. These calculations were carried out in the high-field regime of $a_0 \ll \lambda$ and cannot be directly applied to our low-field case. The previously evaluated $\Delta s \approx 0.06k_B$ in BSCCO at 220 Oe (ref. 22)

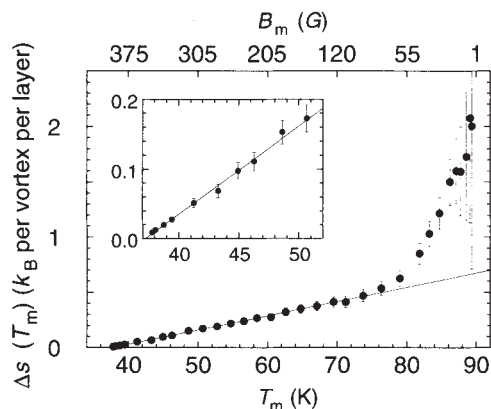


FIG. 6 Entropy change per vortex per layer at the melting transition as a function of T_m . Inset: expanded view near the critical point. Solid lines show linear fit to the data.

is significantly lower than our result of $0.28 \pm 0.03k_B$ reported here at the same field. Another point of reference is the melting of usual solids. In metals a typical value of the latent heat is $1 k_B T_m$ per atom. In BSCCO at $T_m = 83$ K, for example, $1 k_B T_m$ of energy per vortex per layer is similarly required to melt the vortex lattice (although for vortex lines the characteristic vortex length is ϵa_0 and not d as in the decoupling case). However, in regular solids the density of the solid cannot be changed substantially by changing the pressure. The density of the vortex lattice, in contrast, changes by orders of magnitude along the melting line of Fig. 4. The associated latent heat per vortex is also changing by orders of magnitude from zero at high vortex densities to substantial values at low vortex densities close to T_c .

We note that in YBCO the vortex solid is pinned and finite critical currents exist below $B_m(T)$, which cause the observed step in resistivity at the melting transition^{16, 21}. In BSCCO, in contrast, the vortices are unpinned below the transition at elevated temperatures³⁴, and such a resistive step should therefore not be observable, consistent with the experimental results³³. On the other hand, in YBCO it would be very difficult to observe the field step owing to the small $\Delta B/B_m$ ratio and because of vortex pinning below the transition.

Another interesting point is that the observed phase transition in BSCCO occurs at fields that are three orders of magnitude below the mean-field transition at H_{c2} , demonstrating the enhanced role of thermal fluctuations. The theoretical estimates on the basis of the vortex-lattice melting and decoupling transition models give $\Delta B \approx 0.5$ G, in reasonable agreement with the data, but neither of the existing theories can fully describe the temperature dependencies of $B_m(T)$ and $\Delta B(T)$. □

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Crystal structure of the ligand-binding domain of the human nuclear receptor RXR- α

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The crystal structure of the human retinoid-X receptor RXR- α ligand-binding domain reveals a previously undiscovered fold of an antiparallel α -helical sandwich, packed as dimeric units. Two helices and one loop form the homodimerization surface, and hydrophobic heptad repeats participate in stabilizing the fold. The existence of a ligand-binding pocket is proposed that would allow 9-*cis* retinoic acid to interact with different functional modules, including the AF-2 activating domain. Several lines of evidence indicate that the overall structure is a prototype fold of ligand-binding domains of nuclear receptors.

THREE retinoic acid (RARs) and three retinoid-X receptors (RXR- α , β , γ and isoforms) transduce the pleiotropic effects of retinoic acids on morphogenesis, differentiation and homeostasis during embryonal development and postnatal life (refs 1, 2 and references therein). RARs and RXRs belong to the nuclear receptor superfamily whose members act as ligand-inducible transcription regulatory factors. RARs are activated by both isomers of retinoic acid, all-*trans* (*t*-RA) and 9-*cis* (9*c*-RA) retinoic acid, whereas only 9*c*-RA binds to and activates RXRs (reviewed in refs 1 and 2). RARs and RXRs can form homodimers, but heterodimerization greatly increases the efficiency of binding to cognate direct-repeat (DR) DNA response elements, resulting in the formation of anisotropic heterodimer-DNA complexes (refs 3 and 4, and references therein). In addition, RXRs are involved in several other nuclear signalling pathways. Binding to the specific response elements of thyroid hormone, vitamin D₃ and peroxisome proliferator-activated receptors is enhanced by heterodimerization with RXRs (reviewed in refs 1, 2, 5).

As members of the nuclear receptor superfamily, RXRs display a modular structure with five distinct regions (denoted A–E in Fig. 1*a*), of which the DNA-binding domain (DBD; region C) and ligand-binding (LBD; region E) domain exhibit the highest degree of evolutionary conservation^{1,6–10}. So far only the structures of the DBDs of some nuclear receptors have been solved by X-ray crystallography and NMR (refs 11–14 and references therein). Region E must correspond to a multifunctional domain as, in addition to the LBD, it harbours a homo- and a heterodimerization surface, and the ligand-dependent transactivation function AF-2 (reviewed in refs 1, 2 and 8). Understanding how these various functions operate and interact at the molecular level requires both functional and structural analysis. Here we present the crystal structure of the human RXR- α apo-LBD, which corresponds to a novel fold and is likely to be prototypic of the LBDs of the other members of the nuclear receptor superfamily. We discuss the structural basis of ligand binding, homodimerization and transactivation by AF-2.

Structure determination and refinement

The histidine-tagged region DE of RXR- α (hRXR α (DE)¹⁰; Fig. 1*a*) was overexpressed using an *Escherichia coli*/T7 system and purified (Table 1). Crystals of space group *P*6₃22 (*a* = *b* = 110.8 Å, and *c* = 109.9 Å; one molecule per asymmetric unit), were grown in the absence of the 9*c*-RA ligand by vapour diffu-

sion (Table 1). Their limit of diffraction is 2.5 Å, but a full data set could only be measured for the 2.7 Å sphere. The three-dimensional structure was solved by multiple isomorphous replacement (MIR) using two derivatives, xenon (ref. 15 and references therein) and mercury (Table 1). Note that the two binding sites of the xenon atoms are located in a pocket that corresponds to one of the putative ligand-binding sites (B; see below). An initial 5-Å-resolution MIR map revealed clear electron density for α -helices, but not all main-chain connectivities were obvious. Using the graphics modelling program O (ref. 16), a discontinuous polyalanine model was then built into a 3.5-Å-resolution map phased by MIR and solvent-flattening techniques. The map was further improved by combining the phases derived from this partial model with the MIR phases. Several cycles of model building and phase combination gave an unambiguous tracing. A first sequence assignment could be done from the mercury-binding sites (Cys 404 and Cys 432) in the PCMBs derivative. Side chains were then added using omit ($2F_o - F_c$) difference Fourier syntheses. The unambiguous location of the residues required several rounds of manual model building, refinements with X-PLOR¹⁷ and phase combination. A representative portion of the final electron density map of the E region is illustrated in Fig. 2*a*. No reliable model could be built for the N-terminal part of the expressed protein which corresponds to the histidine tag (20 residues) and the 'hinge' region D (24 residues) because it had no clear electron density and was likely to be disordered in the crystal. The final model comprises the unambiguous trace of the LBD (Ser 225 to Thr 462¹⁰; Fig. 1*b*; 238 amino acids, 1,871 atoms). It was refined between 8 and 2.7 Å resolution to the agreement factors R_{cryst} = 0.230 and R_{free} = 0.342 (Table 1). According to all stereochemical parameters tested by the program PROCHECK¹⁸, the quality of the present RXR model is similar or better than that of well refined structures at the same resolution. Only 2 out of the 238 backbone torsion angle combinations (both residues are in loops) occupied unfavourable regions of the Ramachandran (ϕ , ψ) plot.

The α -helical sandwich

The fold of the RXR- α LBD (Fig. 2*b, c*, and see topology in Fig. 1*c*) can best be described as an antiparallel α -helical sandwich with dimensions of 38 × 74 × 25 Å. The α -helical content is very high and agrees well with the results from circular dichroism (W.B. *et al.*, in preparation) and with the secondary structure predictions obtained using the GCG package (University of Wisconsin). Eleven α -helices account for 65% of the domain. They are organized in a three-layer structure, with helices H4, H5,

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TABLE 1 Crystallographic analysis

Data set	Native	PCMBs*	Xe†
X-ray source	LURE	Laboratory	LURE
No. of crystals	1	1	5
Resolution (Å)	2.7	4.0	3.5
No. of unique reflections	9,167	3,544	5,284
Completeness (%)	85	96.2	97.6
Multiplicity	4.8	3.3	5.7
$R_{\text{sym}}(I)$ (%)‡	6.9	7.7	12.6
Reagent concentration		2 mM	20 bars
Soaking time (h)		24	1
MIR analysis (30–3.5 Å)			
No. of sites		2	2
Phasing power (centric/acentric)§		1.3/0.9	2.3/1.4
Mean overall f.o.m.	0.60		
Refinement (8–2.7 Å)			
R_{cryst} (%)¶	23.0		
R_{free} (%)¶	34.2		
Non-hydrogen atoms	1,871		
No. of water molecules	37		
R.m.s. bond length (Å)	0.020		
R.m.s. bond angle (°)	2.48		
Average B-factor (Å ²)**	34.3		

Purification, crystallization and preparation of heavy-atom derivatives: The histidine-tagged, N-terminally truncated human RXR- α (His₆-hRXR α (DE); hRXR α amino acids 200–462), comprising the entire 'hinge' region D and the ligand-binding domain E, was overexpressed in *E. coli* BL21(DE3) bacteria using the T7 system and purified by Ni²⁺-ion affinity chromatography as described (W.B. *et al.*, manuscript in preparation). Using this procedure, 8–10 mg His₆-hRXR α (DE) per litre of culture could be purified to more than 95% homogeneity, as judged from silver-stained gels and immunoblots. NMR spectra were compatible with a folded structure and suggested the presence of hRXR α (DE) dimers; circular dichroism spectra indicated a high content of α -helical structures. Fluorescence-quenching analysis showed that purified His₆-hRXR α (DE) preparations bound 9-*cis* retinoic acid quantitatively (>95%) and with high affinity ($K_d = 13.8 \pm 1.2$ nM; W.B. *et al.*, in preparation). Crystals were grown at 15 °C from 0.45 M ammonium citrate in a buffer consisting of 30 mM PIPES (pH 7.0), 30 mM KCl, 4% glycerol and 5 mM CHAPS using the vapour diffusion method. The droplets were equilibrated against a reservoir containing 1.5 M ammonium citrate and 100 mM PIPES at pH 7.0. Heavy-atom derivatives used for multiple isomorphous replacement were obtained by either soaking the crystals with a mercurial reagent (PCMBs) or placing crystals in a gaseous xenon (Xe) atmosphere during data collection¹⁵. For soaking with PCMBs, all crystals were first stabilised in 2 M ammonium citrate (pH 7.0) and then transferred into 1.8 M ammonium sulphate at the same pH. Data collection, phase determination and refinement: X-ray diffraction data were collected at 15 °C using a refrigerated nitrogen gas delivery system. Synchrotron data were collected at the Laboratoire pour l'Utilisation du Rayonnement Electromagnétique (LURE), Orsay, France ($\lambda = 0.98$ Å) on a MarResearch imaging plate detector using 0.5° oscillations. Laboratory data were collected using either a MarResearch imaging plate detector or a Siemens area detector mounted on a rotating-anode generator ($\lambda = 1.54$ Å). Data were processed using the MarXDS package⁴⁵ and further processed using programs from the CCP4 package⁴⁶. The xenon and mercury sites were located by difference Patterson analysis. Heavy-atom position refinement and phasing were done using the program MLPHARE⁴⁶. MIR phases at 3.5 Å were improved by solvent-flattening using the program SQUASH⁴⁶ and subsequently used for re-refining the heavy-atom parameters. The solvent content in the crystal was calculated as 55% per asymmetric unit, but a lower value (40%) was used to avoid a loss of protein density during the solvent-flattening procedure. The refined model included the ordered part of the protein (entire E domain, 238 amino acids) and 37 solvent molecules. These belong to the first shell of hydration and were located in difference Fourier maps. Individual B-factors were refined as isotropic and restrained. Their values exhibit a normal distribution in the range 20–40 Å², but are higher (50–60 Å²) for a few residues close to the disordered N-terminal region, the H9–H10 loop and the C-terminal end, where higher flexibility or partial disorder is expected. All solvent molecules have a temperature factor lower than 50 Å².

* PCMBs, sodium *p*-chloromercuribenzyldisulphonate.

† Xe, xenon.

‡ $R_{\text{sym}}(I)$ (%) = $\sum_i \sum_j |I_{hki}| - I_{hkl}| / \sum_i \sum_j I_{hkl}$, where i is the observed intensity and $\langle I \rangle$ is the average intensity obtained from multiple observations of symmetry related reflections.

§ Phasing power is $\langle F_H \rangle / \langle \epsilon \rangle$, where $\langle F_H \rangle$ is the r.m.s. calculated heavy-atom structure factor and $\langle \epsilon \rangle$ is the r.m.s. lack of closure.

|| f.o.m., figure of merit.

¶ R_{cryst} (%) = $\sum |F_o| - F_c| / \sum |F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes (all data, cutoff = $F > 2\sigma$).

* R_{free} (%) was calculated using 10% of the reflections, randomly chosen, which were not used in the refinement.

** Average B-factor is the average B-factor for all non-hydrogen atoms in the structure.

TABLE 2 Amino acids identified as part of or close to the ligand-binding pockets of various receptors

Nuclear receptor	Residues identified	Technique	Residues in RXR	Ref.
hAR	Thr 868	NM	Phe 437	31
hER	Asp 426	DM	Asp 347	33
	Gly 521	DM	Cys 432	30
	Cys 530	AL, DM	Leu 441	26
hGR	Met 604	AL	Leu 309	27, 28
	Cys 643	DM	Phe 346	32
	Cys 638	AL	Gly 343	27
	Cys 736	AL	Phe 437	27, 28
hPR	Met 759	AL	Leu 309	28
hTR- β	Gly 345	NM	Ile 318	34
hRAR- α	Gly 301	DM	Ile 318	35

Amino acids are shown that are part of or close to the ligand-binding pockets of the human receptors for androgen (hAR), oestrogen (hER), glucocorticoid (hGR), progesterone (hPR), thyroid hormone (hTR) or retinoic acid (hRAR). Amino acids identified by ligand-affinity labelling (AL) and/or site-directed mutagenesis (DM) are indicated, together with the positions of the corresponding RXR residues deduced from sequence alignment^{9,29}. Comparison with Fig. 4c identifies these residues in the immediate vicinity of the 9c-RA binding pocket B. NM, natural mutation.

H8, H9 and the N-terminal part of H11 sandwiched between H1, H2 and H3 on one side and H6, H7 and H10 on the other (Figs 1c, 2b, c). Two short β -strands (s1 and s2) forming a β -hairpin constitute the only β -structure of the domain. They are located between H5 and H6 in the middle layer and are important as parts of the putative hydrophobic ligand-binding pocket (see below). The tip of the hairpin is inserted between H2 and H3 of the first layer and appears to be responsible for the separation of H1 and H2. Most helices exhibit a regular α -helical conformation, but two remarkable kinks occur at Gly 304 and Gly 443. The kink at Gly 304 generates the two separate helices H4 and H5. The consequence of the kink at Gly 443 in the N-terminal part of helix H11 is that most of H11 points away from the core of the molecule, such that some but not all of the residues important for the activity of the AF-2 activating domain (see below and Fig. 4a) are exposed to the solvent.

Homodimerization interface of RXR LBD

In keeping with the presence of homodimers in the preparations used for crystallization (approximately 50%, as revealed by NMR, light scattering and gel filtration analysis; data not shown), the crystal packing of the unliganded RXR LBD shows strong intermolecular interactions across the two-fold symmetry axis, indicating that the dimer interface matches up with the crystallographic axis. Each RXR LBD monomer contributes 11% (1,333 Å²) of its solvent-accessible surface to the dimerization interface, typical of specific protein–protein interactions. For example, the 1,630 Å² interface of alcohol dehydrogenase dimers represents 11.2% of the accessible surface area¹⁹. The dimer interface is formed mainly by helices H10 and, to a lesser extent, H9 and the loop between H7 and H8 (Fig. 3a, b). The two monomers form a rotationally symmetric dimer with the two C-terminal 'activation helices' H11 pointing away from each other with an angle of roughly 45° relative to the symmetry axis (Fig. 3a). In contrast, the N-terminal ends of the LBD monomers are in much closer proximity with one another, perhaps to facilitate the formation of a second dimerization interface between two RXR DBDs^{3,14}. As two RXR DBDs bind their cognate DR1 response element in a head-to-tail configuration, a flexible hinge region D has to be postulated to allow for a rotation of the DBD domains while keeping the 2-fold-related symmetrical contact of the LBD dimer.

The most prominent interactions of the dimer interface in the LBD are illustrated in Fig. 3b, c and d. They comprise hydrophobic van der Waals contacts between pairs of Leu 419, Pro 423

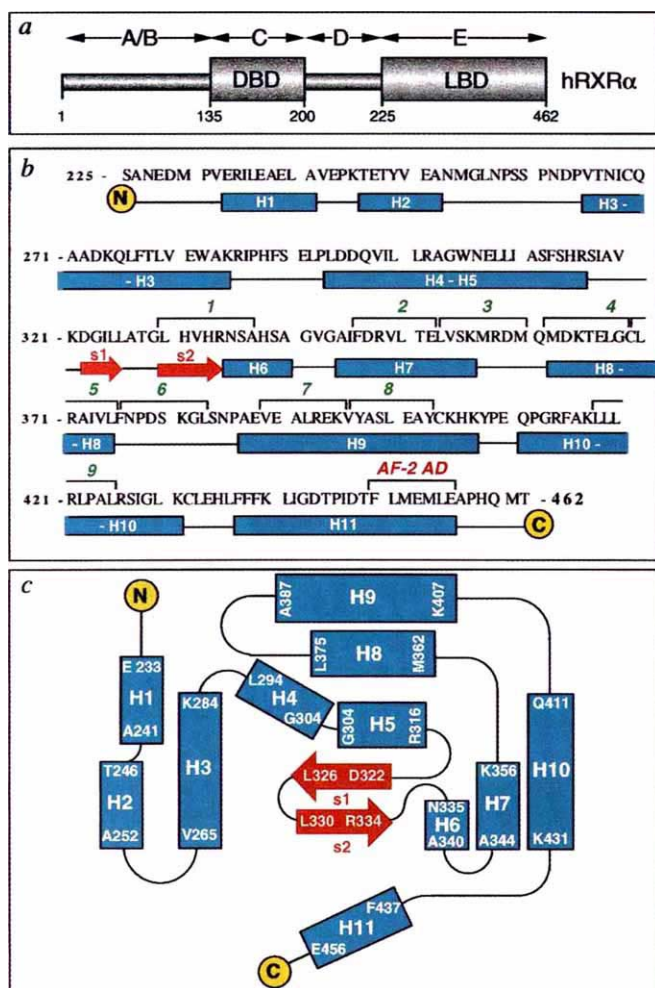


FIG. 1 a, Schematic representation of the human RXR- α (hRXR- α). The DNA-binding (DBD; region C) and ligand-binding (LBD; region E) domains are boxed. b, Amino-acid sequence of RXR- α LBD, with the secondary structural elements indicated by blue rectangles (α -helices; H1–H11) and red arrows (β -strands; s1 and s2). Heptad repeats²³ (green numbering 1 to 9) and the AF-2 activating domain (AD; red)^{35,40} are indicated above the sequence. c, Topology: the N and C termini of the RXR- α LBD are labelled, as are the residues at the beginning and end of each α -helix or β -strands. Red arrows represent β -strands (s) and blue rectangles represent α -helices (H).

and Leu 430 (contacts H10–H10), as well as hydrophilic interactions involving Asp 379 (loop H8–H9) and Glu 390 (H9) of one monomer with Arg 421 (H10) and Lys 417 (H10) plus Arg 358 (loop H7–H8), respectively, of the other monomer. Within each monomer, an additional interaction between Lys 417 (H10) and Asp 359 (loop H7–H8) buttresses the hydrogen-bond network and equilibrates the charge distribution. Two further salt bridges formed by Glu 401 and Lys 405 link the two ends of the symmetrically related H9 helices. With the exception of Leu 430, which is replaced by serine, all of these residues are conserved in COUP-TFI/ear-3 and COUP-TFII/arp-1 (ref. 9), which homodimerize efficiently. Any mutation at the position of Pro 423 would alter the helical axis of H10, and thus affect both RXR homodimerization and the internal hydrophobic pocket that this helix forms with H8 and H9. Indeed, mutation of Pro 423 to threonine reduces the efficiency of RXR homodimerization²⁰.

Homodimerization of RXR is due to the presence of a weak dimerization function in the RXR DBD^{3,14} and a strong dimerization function in the RXR LBD^{20–22}. Nine conserved hydrophobic heptad repeats (Fig. 1b) in nuclear receptor LBDs have

been postulated to correspond to potential dimerization surfaces²³. Mutational analyses have identified residues in heptad9 as critical for both homo- and heterodimerization of RXR^{20,24}. As helix H10, a part of the dimer interface in the crystal, encompasses the ninth heptad repeat (Leu 418 to Leu 425), the interface observed in the crystal is most probably identical to the one formed in solution. None of the other heptad repeats is involved in RXR dimerization. Interestingly, the hydrophobic heptad repeat residues proposed to be important for dimerization²³ do not contribute directly to the homodimeric interface. Indeed, in the case of heptad 9 these residues pack towards H5, H8 and H9 (Fig. 4a). Similarly, the crystal structure of the RXR LBD clearly reveals that the 'hydrophobic' residues of heptads 4, 5 (H8) and 6 stabilize the LBD architecture through intramolecular interactions with residues of heptads 7 and 8 (H9), whereas some residues of heptad 1 (s2 and H6) interact with heptads 2 and 3 (H7) (Fig. 4a).

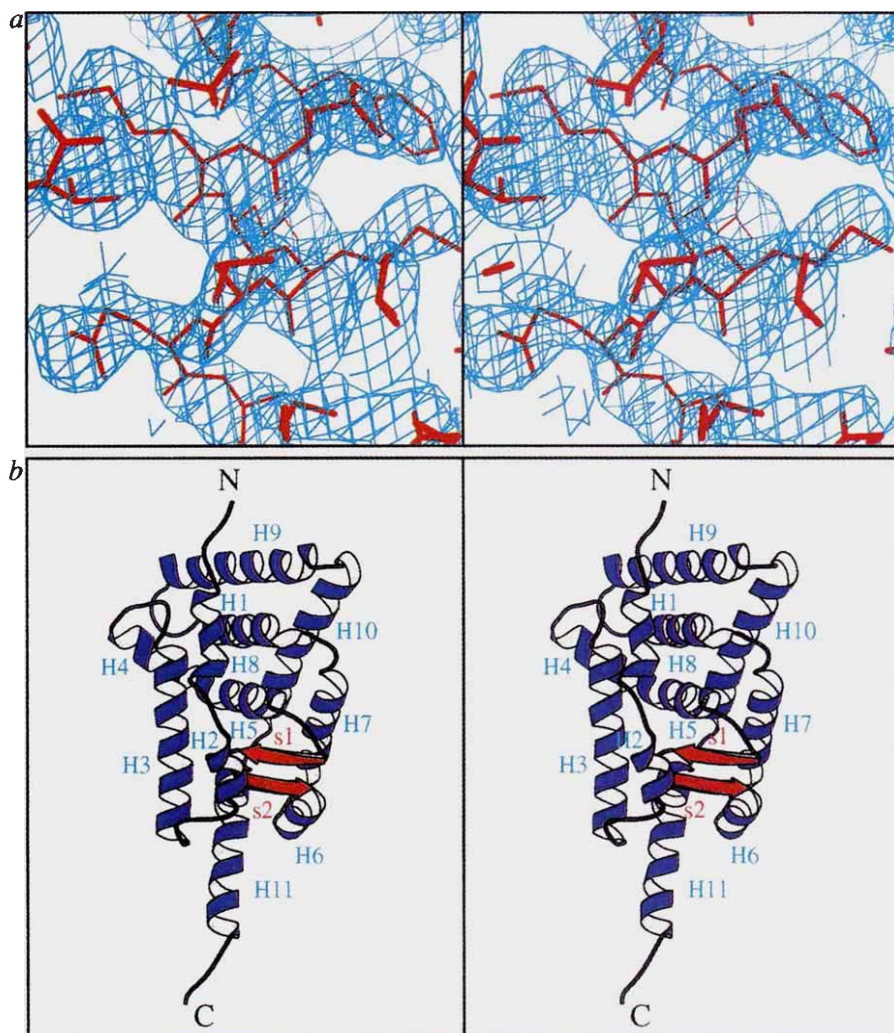
The putative ligand-binding site

A search for potential binding pockets for 9c-RA revealed two large hydrophobic cavities (referred to as A and B), located in the vicinity of and partly formed by the s1, s2 β -hairpin. These cavities are separated by H5, with Phe 313 and Leu 309 participating in the formation of the pockets A and B (Fig. 4b). Using the graphics modelling program O (ref. 16), 9c-RA could be manually docked in both cavities, requiring only minor conformational adaptation of the side chains (Fig. 4c). Pocket A, formed by s1, s2, H1, H3, H5 and the N-terminal part of H11 is accessible to the solvent. Pocket B is an elongated hydrophobic cavity formed by H5, s1, s2 and H7, together with both the C-terminal part of H10 and the N-terminal region of H11 (Figs 4b, c and 2b). Two lines of evidence support the idea that pocket B is the actual binding site: (1) it resembles that of the epididymis retinoic-acid-binding protein E-RABP²⁵, with a set of four aromatic amino acids (Phe 313, Phe 346, Phe 437, Phe 438) surrounding the 9c-RA β -ionone ring. However, *t*-RA binds E-RABP in a very large and deep pocket at the centre of a β -barrel²⁵, whereas in RXR, 9c-RA would bind in a cavity mainly surrounded by α -helices; and (2) the idea correlates well with crosslinking and mutational data obtained with other nuclear receptors (see below). In support of an implication of either binding site A or B, a deletion of 30 C-terminal amino acids, removing four of the residues of pocket B (Phe 437, Phe 438, Leu 441, Ile 442) and two of the residues of pocket A (Leu 441, Ile 442) abrogates ligand-induced protease sensitivity²⁴, whereas RXR truncated at amino acid 448 still binds the ligand (our unpublished data).

Ligand-affinity labelling has identified residues in the LBDs of the oestrogen (ER), progesterone (PR) and glucocorticoid (GR) receptors that belong to the steroid-binding pocket^{26–28}. An alignment of nuclear receptor LBDs^{9,29} reveals that all of the corresponding residues of RXR surround the putative 9c-RA binding pocket B (Table 2 and Fig. 4c). In addition, mutation of some of these residues in the LBDs of several nuclear receptors can alter the ligand-binding properties without impairing other functions of the LBD^{26,30–35} (Table 2). Together these observations support the conclusions that cavity B probably corresponds to the RXR ligand-binding pocket, and that a similarly located binding pocket may exist in steroid, thyroid hormone (TR) and retinoic acid receptors.

Protease clipping experiments have suggested that 9c-RA binding induces a structural change in the LBD of mouse RXR- β (ref. 24), so, it is surprising that only minor structural changes are required to accommodate the ligand in pockets A or B. In this respect, it is important to note that the exact amplitude of the conformational alteration of the RXR LBD cannot be estimated from protease clipping data, and that ligand binding may trigger a subsequent conformational modification, namely of H11, without gross alteration of the binding pocket itself. This could account for the effect of ligand binding on transactiv-

FIG. 2 a, Stereo view of the 2.7 Å resolution ($2F_o - F_c$) map, contoured at 1.2 standard deviation, with a stick representation of the atomic model for the refined structure superimposed (drawing generated by MINIMAGE⁴⁷). The picture shows the AF-2 AD which encompasses the sequence 450-FLMEMLE-456. b, Stereo drawing of the hRXR- α LBD backbone. c, Ribbon drawings of two views (120° rotation) of the hRXR- α LBD. Colour coding is according to position in the sequence, starting with red at the N terminus (Ser 225) towards dark blue at the carboxyl end of the chain (Thr 462). Individual helices (H1 to H11) and β -strands (s1 and s2) are indicated.

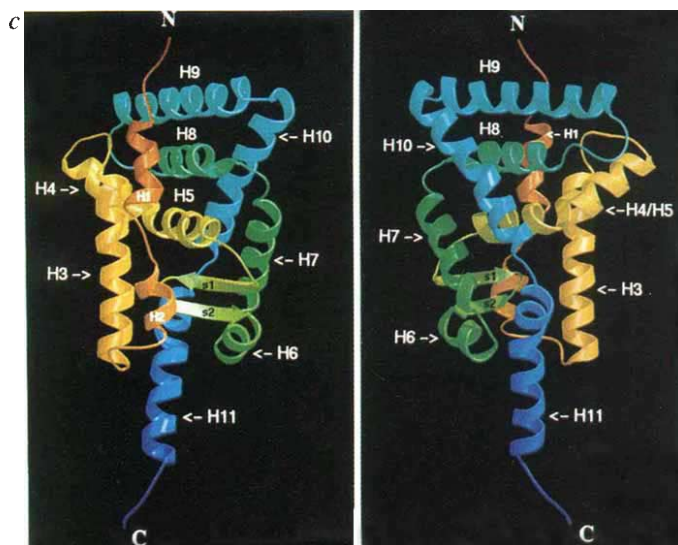


ation by AF-2 (see below). However, penetration of ligand into either cavity A or B requires at least some conformational changes of amino-acid side chains.

The C-terminal activation domain

The LBD of RXR contains a ligand-inducible activation function³⁶, termed AF-2 by analogy with the AF-2 activation functions originally described for steroid receptors (reviewed in refs 1, 2, 6, 8). An AF-2 activating domain has been characterized in the C-terminal part of the LBDs of RAR, RXR, TR and oestrogen receptor (ER). Its main features are conserved between all known transcriptionally active members of the nuclear receptor superfamily, but it is deleted in *v-erbA* and altered in some orphan receptors that lack an AF-2 activity^{24,35,37-39}. The AF-2 activating domain, which in the case of TR, RAR and RXR autonomously transactivates and transcriptionally interferes (squashes)^{24,35,39}, may form an amphipathic α -helix³⁷.

The RXR- α AF-2 activating domain, which encompasses the sequence 450-FLMEMLE-456 (refs 24, 35, 40) indeed adopts a helical structure, corresponding to the C-terminal part of helix H11 (Figs 1b and 4a). This 'activation helix' extends from the core of the LBD, pointing away from the dimer axis at an angle of about 45° (Fig. 3a). Thus, the position of the AF-2 activating domain in the RXR- α LBD appears to be optimal for facilitating interactions with putative transcriptional intermediary factors^{41,42}, like the recently identified TIF1⁴⁰. But residues Phe 450 and Glu 453, which are critical for both TIF1 binding and transactivation⁴⁰, have limited accessibility in the unliganded RXR- α LBD. Phe 450 is close to Pro 264 (at the start of H3),



which itself is in van der Waals contact with Ile 447 of the activation helix H11, and Glu 453 and Asn 262 (in the loop between H2 and H3) are apparently hydrogen-bonded. Thus, an alteration of the present LBD structure may be necessary to allow TIF1 binding. Two distinct mechanisms have been discussed that can lead to ligand-dependent transactivation by AF-2 (refs 35, 40). Either the unliganded LBD harbours an inactive or intramolecularly 'masked' AF-2 (as appears to be the case here)

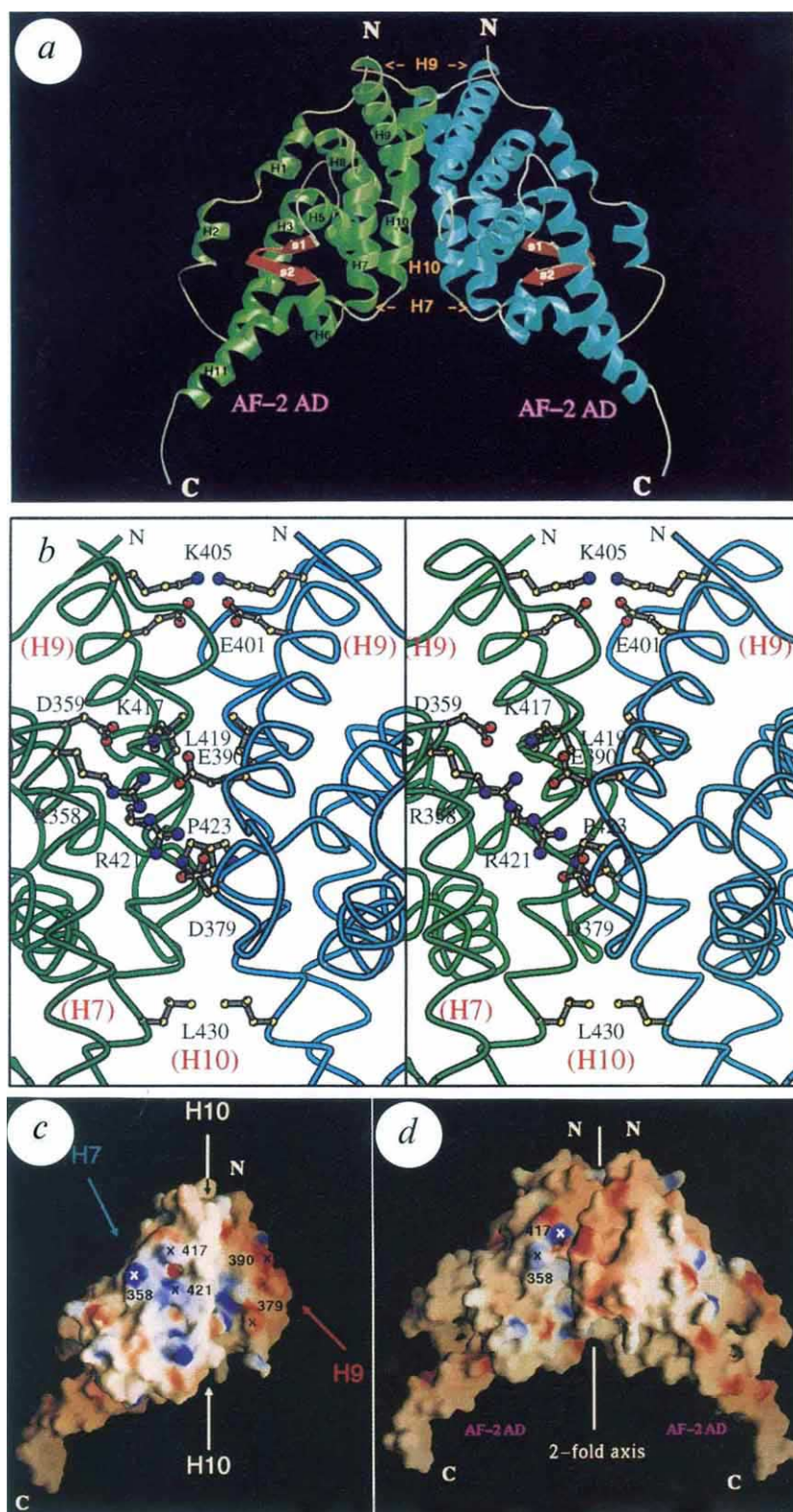


FIG. 3 **a**, Three-dimensional structure of the RXR LBD homodimer. View of the homodimer showing the interface formed by the helices H9, H10 and the loop H7–H8. Monomers are represented in blue and green. The antiparallel β -strands of both monomers are in red. The viewing direction is perpendicular to the two-fold symmetry axis that lies vertically in the figure plane. **b**, Stereo view magnification of the dimer interface showing the most prominent interactions of the side chains that stabilize the dimer. **c**, Surface representation of the monomeric unit showing the electrostatic potential. The charge distribution calculated using the program GRASP⁴⁸ is represented as red for negative and blue for positive charges. The residues (numbers indicate positions in hRXR- α) establishing hydrophilic contacts between the two subunits are indicated by crosses. Helix 10 (white arrow, H10), which contributes most of the hydrophobic contacts, comes out as a white streak. Flanking regions of H10 (blue and red arrows) bear complementary charged residues. **d**, View of the hRXR- α LBD dimer. Orientation and colour code are as in **c**.

unpublished data). Perhaps the interactions between H11 and both H3 and the loop between H2 and H3 are disrupted upon ligand binding, making the AF-2 activating domain accessible for protein–protein interactions. Crystallization of the 9 α -RA-ligated RXR LBD will be necessary to validate this model, as well as to explain the structural basis for the inability of RXRs to bind *t*-RA.

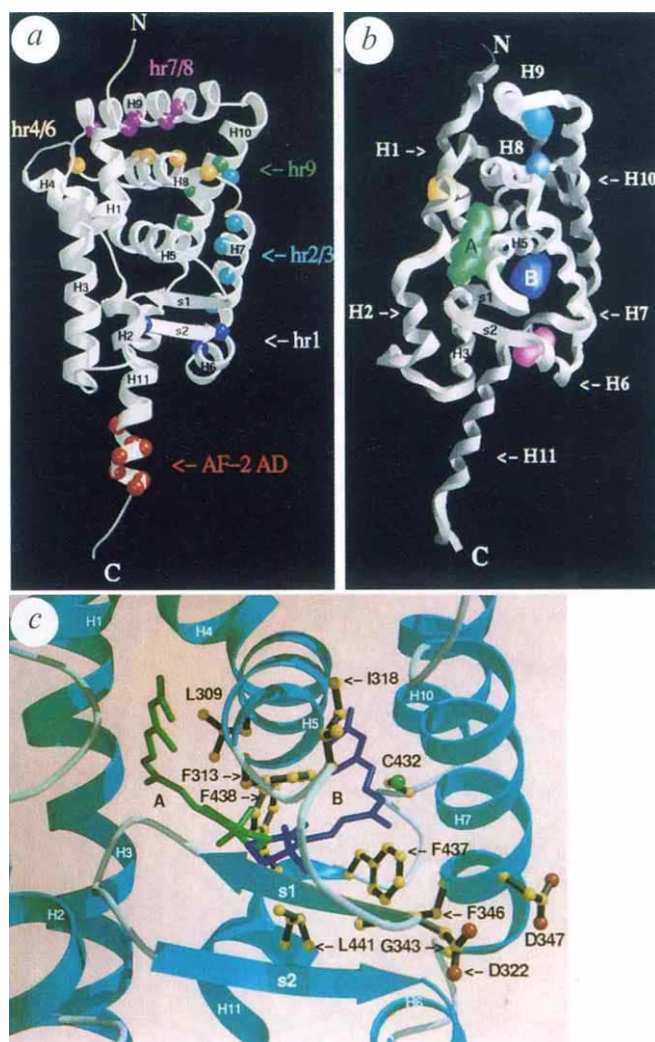
A prototype fold

Several lines of evidence indicate that the present three-dimensional structure of the RXR LBD corresponds to a prototypic fold with structural modules that appear to be conserved within the nuclear receptor superfamily. (1) Sequence alignments indicate that the existence of most of the H1–H11 α -helices seen here can be predicted in many nuclear receptors, and insertions/deletions are usually seen in linker regions (refs 9, 23, 29, and our unpublished data). The three most conserved domains²⁹ overlap with H3, H4, H7, H8 and H10 of RXR- α LBD. Furthermore, a convincing alignment⁹ based on the conservation of α -helical heptad repeats²³ predicts the conservation of H3 to H10. (2) Intramolecular interaction involving the heptad repeats appears to be crucial for the stabilization of the architecture of the RXR LBD. The conservation of these repeats within the nuclear receptor superfamily²³ therefore indicates that the structural organization of the various LBDs is similar. (3) Heptad 9, which is part of the helix H10 that contributes to the homodimerization interface of RXR (see above), is highly conserved among nuclear receptors that form homo- and/or heterodimers and was correctly predicted to adopt an α -helical structure^{23,30}. As in the case of RXR- α (see above), mutations in at least three receptors,

and its activity is induced through conformational change (ref. 24 and references therein) upon ligand binding, or AF-2 is present in an active conformation within the unliganded LBD and its activity is blocked *in vivo* by intermolecular interaction with a 'repressor' that is released upon ligand binding. Although this latter possibility cannot be ruled out, we consider it unlikely because efficient interaction *in vitro* between the bacterially expressed purified RXR LBD and the putative transcriptional intermediary factor TIF1 requires 9 α -RA (ref. 40, and our

ER, TR and RAR, have shown that heptad 9 contains residues critical for dimerization^{22,30,35}. It is thus likely that the dimerization interfaces of other nuclear receptors are related to the one described here for the RXR LBD. However, deletion studies suggest that the homo- and heterodimerization surfaces of RXR are not fully superimposable²⁴. (4) A number of residues, identified as critical for ligand binding by other nuclear receptors, have locations similar to those that surround the putative 9 α -RA binding pocket B (Fig. 4c and Table 2). As this pocket is

FIG. 4 The various structural and functional elements in the three-dimensional model of the RXR LBD (see text for details). *a*, Heptad repeats and transactivation domain. The RXR amino acids at positions 1, 5 and 8 of the 9 heptad repeats (hr) proposed to form the dimerization surface²³ are illustrated as CPK representations (only the α -carbon is shown for clarity; blue, hr1; cyan, hr 2 and 3; yellow, hr 4 to 6; violet, hr 7 and 8; green, hr 9). α -Carbons of the RXR residues constituting the transactivation domain AF-2 AD^{35,40} are red. *b*, GRASP⁴⁸ representation of the various cavities found in the unliganded RXR- α LBD. The two potential 9c-RA binding sites A and B are displayed in green and blue, respectively. The remaining cavities are too small to accommodate 9c-RA and exhibit an unfavourable hydrophobic environment. *c*, View of the putative RXR ligand binding pockets with 9c-RA (in green and blue for sites A and B, respectively). The helices and the β -hairpin (s1 and s2) forming the binding cavities are indicated, together with the side chains of RXR residues corresponding to amino acids of other nuclear receptors that are involved in ligand binding (with the exception of D322). For the role of F313 and F438, see text. Figures 2, 3a, b, 4a, c were drawn using RASTER3D⁴⁹ and/or MOLSCRIPT⁵⁰.



formed from several regions of the RXR LBD, the similarity of these locations argues for a conservation of the overall architecture of nuclear receptor ligand-binding pockets. In this respect it is notable that most of the residues that constitute the α -helices H3–H11 of the RXR LBD are conserved in the *Drosophila* RXR

homologue, the orphan receptor Usp, which heterodimerizes with the receptor for the invertebrate moulting steroid hormone ecdysone⁴³. Clearly, the present architecture of the RXR LBD emerged before the divergence of arthropod and vertebrate lineages, which occurred more than 550 million years ago. □

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Nitrogen isotope abundances in the recent solar wind

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ALTHOUGH lunar crystalline rocks are essentially devoid of nitrogen, the same is not true of the lunar regolith. The nitrogen contents of individual regolith samples (which can be as high as 0.012% by mass) correlate strongly with abundances of noble gases known to be implanted in the lunar surface by solar radiation, indicating that lunar regolith nitrogen is also predominantly of solar origin¹. The large variability in $^{15}\text{N}/^{14}\text{N}$ ratios measured in different regolith samples may thus reflect long-term changes in the isotopic composition of the solar radiation¹. But attempts to explain these variations have been hampered by the lack of any firm constraint on $^{15}\text{N}/^{14}\text{N}$ in the present solar wind. Here we report measurements of nitrogen isotopes from two lunar samples that have had simple (and relatively recent) exposure histories. We find that nitrogen implanted in the lunar surface during the past 10^5 to 5×10^7 years has a $^{15}\text{N}/^{14}\text{N}$ ratio approximately 40‰ higher than that in the terrestrial atmosphere, which is substantially lower than most previous estimates^{2,3}. This isotopic signature probably represents the best measure of $^{15}\text{N}/^{14}\text{N}$ in the present-day solar wind.

Many explanations have been advanced to account for the long-term variation in $^{15}\text{N}/^{14}\text{N}$ (refs 1, 3–6), but none has yet been accepted as satisfactory. Discrimination between competing explanations would be aided by knowledge of $^{15}\text{N}/^{14}\text{N}$ in the recent, or present-day, solar wind. Thus, some models identify the solar wind as the heavier of two isotopically distinct components that can generate the observed variability as a result of variation in their mixing ratio^{4,5}, whereas another model attributes the variation to fractionation on the Moon of an isotopically light solar wind⁶. Because of the low abundance of ^{15}N , no spacecraft-based measurement of the present-day solar N isotopic signature has the necessary precision. Estimates based on lunar-sample data have ranged from about +50‰ (ref. 7), relative to the terrestrial atmospheric value, to over +200‰ (ref. 8), with several clustering around +110‰ (refs 2, 3, 9). Furthermore, those estimates have tended to rely on assumptions that are currently difficult to verify, so that a direct measurement would be desirable. We report here the results of two independent determinations based on analysis of well-characterized lunar samples known to have had simple recent surface exposures.

Our first experiment⁷ employed clean, hand-picked plagioclase grains separated from 67601, an Apollo 16 soil collected on the rim of North Ray Crater, formed by impact 49 Myr ago¹⁰. North Ray soils are known to contain regolith materials exposed before 49 Myr, laterally transported subsequent to the North Ray event¹¹. However, we expect only a minor contribution from such material among large, fresh plagioclase grains, such as prevail in the crater ejecta. Consequently, the solar-wind loading in large plagioclase grains can be constrained to the past 49 Myr. (More elaborate models can be contrived in which very different radiation histories can be imputed to these grains. However, in other respects 67601 is a typical North Ray rim soil and there are no independent grounds for attributing an anomalous history to its constituent grains.) Nitrogen isotopic and abundance data for a 175–250 µm grain-size fraction of this sample are given in Fig. 1a. The release pattern for stepwise pyrolysis is fairly typical for lunar regolith^{1,12}, with the main release of N between about

700 and 1,050 °C showing a progressive decrease in $^{15}\text{N}/^{14}\text{N}$ with increasing pyrolysis temperature. This phenomenon is tentatively attributed to release of N residing at progressively greater depth. The $\delta^{15}\text{N}$ maximum at relatively low extraction temperature is generally taken to characterize solar-wind N. Accordingly, the low-temperature $\delta^{15}\text{N}$ maximum, which yields a value of $+(48 \pm 7)\%$, represents an average composition for solar-wind N implanted during the past 49 Myr.

Our second experiment employed a sample taken from the top surface of 68815, a well-documented Apollo 16 rock excavation from South Ray Crater 2 Myr ago¹³. The pitted surface of this sample was carefully covered and then cleaved from the main mass, yielding two pieces containing original surface. A subsurface fragment was used for reference purposes. The smaller surface piece was used in a pilot experiment to provide a rough measure of N release, thereby permitting optimization of the temperature steps used for pyrolysis of the larger surface piece, and suggesting an additional combustion step. It also provided material for a replicate surface analysis. The subsurface fragment was analysed as a control to provide N and noble-gas components of non-solar origin (terrestrial contamination, indigenous lunar N and spallogenic N), yielding individual temperature steps with N of uniform isotopic composition ($\delta^{15}\text{N} = 8 \pm 3\%$). The surface-sample data were corrected for contributions from these components by subtracting the subsurface data from those measured for the surface sample. Because the relative abundances of terrestrial contamination (surface-correlated) and indigenous and spallogenic N (volume-correlated) were not known beforehand, it was not clear what fractions of the subsurface data should be treated as surface- and volume-correlated. As the areas of the subsurface and larger surface pieces were similar, and the corrected isotopic data of the smaller surface piece (of different shape) agreed well with those for the larger piece, we treated this blank correction as entirely volume-correlated. Those corrections ranged from 11 to 38% of the measured N, excluding the last two, minor steps (see Table 1). (If, as the other extreme, all corrections were to be treated as 100% surface-correlated, the magnitudes of those corrections would decrease by a factor of about three, increasing N contents for the surface sample by about 14% and $\delta^{15}\text{N}$ values by a few per mil.)

To minimize possible contamination by laterally transported fine lunar soil, surface samples were rinsed briefly with acetone. Care was taken to avoid including sawn faces created when the sample was initially cut out of the rock by the Lunar Sample Curator. The original, space-exposed surface was unambiguously identifiable by the presence of impact pits and a patina

TABLE 1 Nitrogen concentrations and isotopic compositions from the surface of lunar rock 68815

Temp. step (°C)	Measured		Calculated	
	[N] (ng g ⁻¹)	$\delta^{15}\text{N}$ (‰)	[N] (ng cm ⁻²)	$\delta^{15}\text{N}$ (‰)
450 comb	97.9	$+5.5 \pm 3.5$	65.8	$+9.9 \pm 9.7$
550 pyr	38.7	$+8.1 \pm 2.7$	58.2	$+8.6 \pm 3.3$
650 pyr	39.7	$+10.1 \pm 4.7$	60.1	$+10.6 \pm 5.8$
750 pyr	48.9	$+34.2 \pm 2.8$	77.2	$+38.6 \pm 3.3$
700 comb	38.2	$+5.5 \pm 4.2$	63.1	$+5.8 \pm 4.8$
850 pyr	61.3	$+29.4 \pm 1.8$	98.9	$+32.6 \pm 2.1$
950 pyr	34.2	$+27.9 \pm 4.3$	39.3	$+39.6 \pm 6.9$
1,050 pyr	25.0	$+7.1 \pm 4.3$	10.0	$+7.7 \pm 20$
1,170 pyr	55.7	$+19.8 \pm 3.7$	9.5	$+127 \pm 60$
Total	440	$+16.3$	482	$+23.2$

Concentrations, as measured, and as calculated for implanted N after correction, in the large (0.64 cm²; 1.186 mg) surface piece of lunar rock 68815, with corresponding isotopic compositions, expressed as deviations in parts per mil relative to terrestrial atmospheric air. Uncertainties in concentration are $\pm 10\%$ and those in $\delta^{15}\text{N}$ correspond to 95% confidence limits.

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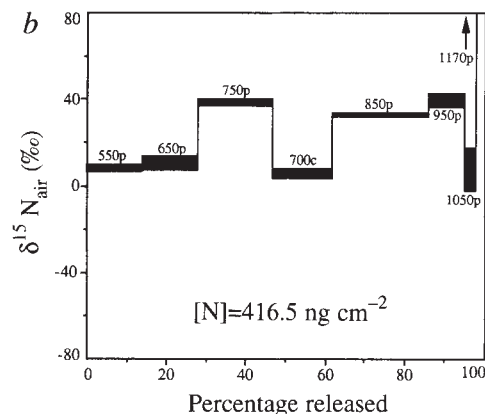
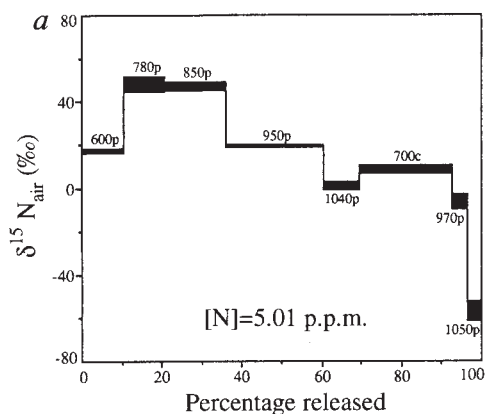


FIG. 1 *a*, Nitrogen isotopic signatures in stepwise release from 67601 plagioclase. This coarse fraction (175–250 μm) is used here because it is least contaminated with older regolith laterally transported from neighbouring terrain. Data for other grain-size fractions are given in ref. 7. A few heating steps were carried out in 5 torr of pure O_2 (combustion). The pyrolysis (p) step at 1,040 $^\circ\text{C}$ was followed by a combustion (c) step at lower temperature (700 $^\circ\text{C}$) releasing an isotopically distinct component, and two further pyrolysis steps also revealed an additional minor component. The plateau at 780 and 850 $^\circ\text{C}$ is taken as solar-

wind N implanted in the past 49 Myr. *b*, Nitrogen isotopic signatures (see Table 1) in stepwise release from a surface sample (0.637 cm^2) of anorthositic rock 68815. The isotopically uniform component in the 750, 850 and 950 $^\circ\text{C}$ pyrolysis (p) steps, which coincides with release of solar-type noble gases, is identified as recently implanted (<2 Myr) solar N. Note the distinct signature of N released in the 700 $^\circ\text{C}$ combustion (c) step, and the release of a heavy component above 1,050 $^\circ\text{C}$ where a contribution from cosmic-ray produced ^{15}N is expected.

characteristic of such lunar-surface material. Similar samples derived from the same rock have been analysed in the past for several lunar-surface phenomena, such as the presence of cosmogenic nuclides^{14,15} and stopped solar-flare noble-gas ions¹⁶.

In addition to N abundance and isotopic composition, we obtained concentration and isotopic data for neon in both surface pieces and for xenon in the larger one. The N data in Table 1 are those for the larger surface piece; the smaller pilot sample yielded a $\sim 40\%$ greater surface concentration of N of identical isotopic composition. Both surface pieces revealed the same $^{14}\text{N}/^{20}\text{Ne}$ ratio, suggesting that the apparently lower concentration in the larger piece is due to uncertainties in the surface-area estimation for the smaller piece, or to differences in erosion or dust coverage on the lunar surface, or to varying damage of the outermost surface during terrestrial handling.

With the exception of the lowest temperatures, the release pattern for the pyrolysis steps (Fig. 1*b*) is dominated by a single component with a $\delta^{15}\text{N}$ value of $+(38 \pm 6)\%$. Given the temperature range in which it is released, 750–950 $^\circ\text{C}$, and its association with noble gases, we interpret this component as implanted solar wind. It is noteworthy that the release profile for 68815 differs from that for 67601 plagioclase, and essentially all previously analysed lunar-regolith samples, in that $\delta^{15}\text{N}$ shows no systematic decrease in the range 850–950 $^\circ\text{C}$ (see Fig. 1*a*). If the normal decrease reflects presence of a more deeply implanted component¹, this suggests that such a component may be missing from 68815. Alternatively, the higher-temperature component may result in some way from the multiple exposures experienced by typical regolith grains but not by 68815. Yet another possibility is that in 68815 the isotopic difference between shallow and deep components is minimal, which is consistent with the apparent long-term evolution of that difference (see Fig. 4 in ref. 1). However, the lack of an isotopically light signature at moderately high extraction temperatures does not vitiate our identification of solar-wind N at lower temperatures.

The markedly different $\delta^{15}\text{N}$ value released by the 450 (not plotted) and 700 $^\circ\text{C}$ combustion steps is suggestive of a non-solar component; these data will therefore not be included in the following discussion.

The Ne concentrations are in agreement with those measured previously in this laboratory for a different sample of this rock¹⁶. The measured $^{14}\text{N}/^{20}\text{Ne}$ ratio, 85 ± 9 , is nearly two orders of magnitude greater than the solar value¹⁷ but is fairly typical for a plagioclase-dominated Apollo 16 regolith sample.

The Xe isotopic data for 68815, like those of a typical regolith sample, carry the signature of solar Xe. Lunar regolith is generally characterized by a $^{14}\text{N}/^{132}\text{Xe}$ ratio in the range $(4\text{--}10 \times 10^6)$ (ref. 18), significantly greater than the solar estimates of 2.5×10^6 (ref. 17) and 1.5×10^6 (ref. 19). Using only the (solar-type) N released in the 750, 850 and 950 $^\circ\text{C}$ pyrolysis steps for 68815 yields a $^{14}\text{N}/^{132}\text{Xe}$ ratio $\sim 3.0 \times 10^6$. (Note that pyrolysis to 1,170 $^\circ\text{C}$ may not have released all of the N and Xe.)

The significance of the N/Xe ratio lies in its potential ability to discriminate between competing theories that seek to explain the long-term trend in $^{15}\text{N}/^{14}\text{N}$. Several such theories attribute the variation of $^{15}\text{N}/^{14}\text{N}$ in the regolith to variable mixing of two end-member components, one being an isotopically invariant solar wind, the other being a hypothetical non-solar component, variously identified as lunar indigenous N (refs 5, 12), meteoritic N (ref. 20), or N from the terrestrial exosphere⁴. In such models, the greater-than-solar N/Xe ratio characteristic of lunar regolith is taken as signalling the presence of non-solar N, even though variations in that ratio do not correlate with variations in $^{15}\text{N}/^{14}\text{N}$ (ref. 1). The observation that the directly implanted surface of 68815 yields the lowest N/Xe ratio found on the lunar surface is consistent with either the presence of a non-solar N component in the regolith or the loss of Xe during regolith processing¹⁸. A ratio for 68815 exceeding the solar values also implies either uncertainties in the solar abundance estimates or partial loss of Xe during implantation. However, we note that constant Ar/Kr and Kr/Xe ratios observed during stepwise etching of regolith minerals preclude loss of those gases by processes, such as diffusion, that cause elemental fractionation among them²¹.

The concentration of N in the surface pieces corresponds to a solar-wind irradiation lasting $\sim 10^5$ years, assuming the present-day solar-wind proton flux, a solar N/H ratio and 100% implantation efficiency. This duration is distinctly shorter than the nominal exposure age of the rock, 2 Myr, presumably owing to erosional processes either on the lunar surface or during sample return and handling. The measured value is broadly consistent with an erosion rate close to that calculated for the solar-wind sputtering rate on the lunar surface, $0.0024 \text{ \AA yr}^{-1}$ (ref. 22). This is the first direct measurement of surface concentration of solar-wind N in a lunar sample, previous analyses having been of particulate material, for which specific surface area had to be inferred.

We conclude that solar N implanted in the lunar surface during the past $>10^5$ years is characterized by a $\delta^{15}\text{N}$ value of $+(38 \pm 6)\%$ relative to the terrestrial atmosphere. The data for 67601 plagioclase are consistent with this value within uncertainties, leading to a weighted average of $+(40 \pm 8)\%$ for recently implanted solar N. This signature is not inconsistent with the long-term trend observed in regolith N (ref. 1). Substantially heavier N, with $\delta^{15}\text{N}$ values in the range $+100$ to $+160\%$ (refs 2, 20), observed in samples of somewhat greater antiquity, apparently do not represent the recent solar radiation, suggesting that $^{15}\text{N}/^{14}\text{N}$ ratios in recorded solar-wind particles may have been significantly greater during the past 1 Gyr, after increasing by some 400‰ during the preceding 1 Gyr (ref. 1). Thus, one may speculate that the long-term trend in the $^{15}\text{N}/^{14}\text{N}$ signature, rather than being simply a linear increase with time as originally proposed²³, has experienced two excursions, to minimum ($<-280\%$) and maximum ($>+160\%$) $\delta^{15}\text{N}$ values, respectively, in a roughly 3.5-Gyr time frame. Systematic isotopic studies of the kind described here offer a rare opportunity to contribute to the study of solar history on a billion-year timescale. □

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Strong second-harmonic generation from centrosymmetric dyes

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SECOND-harmonic generation (SHG)—the frequency doubling of light—requires materials with a non-centrosymmetric structure that gives rise to a large second-order nonlinear optical susceptibility. For molecular materials, it is widely assumed that the molecular structure, as well as the packing arrangement, must also be non-centrosymmetric, unless a magnetic dipole^{1–3} or an electric quadrupole⁴ contributes to the bulk susceptibility. Consequently, most studies have focused on dipolar molecular systems in which the optical nonlinearities arise from intramolecular charge transfer^{5–8}. But the criteria for SHG may also be satisfied by centrosymmetric molecules if they aggregate in a non-centrosymmetric manner and there is a contribution to the bulk susceptibility from intermolecular charge transfer. Here we report the nonlinear optical properties of a series of centrosymmetric dyes based on a squaraine template, which we ascribe to such an effect. Monolayer films of these molecules deposited by the Langmuir–Blodgett (LB) technique show surprisingly large SHG, which compares favourably with the highest hitherto reported for LB monolayers of non-centrosymmetric molecules.

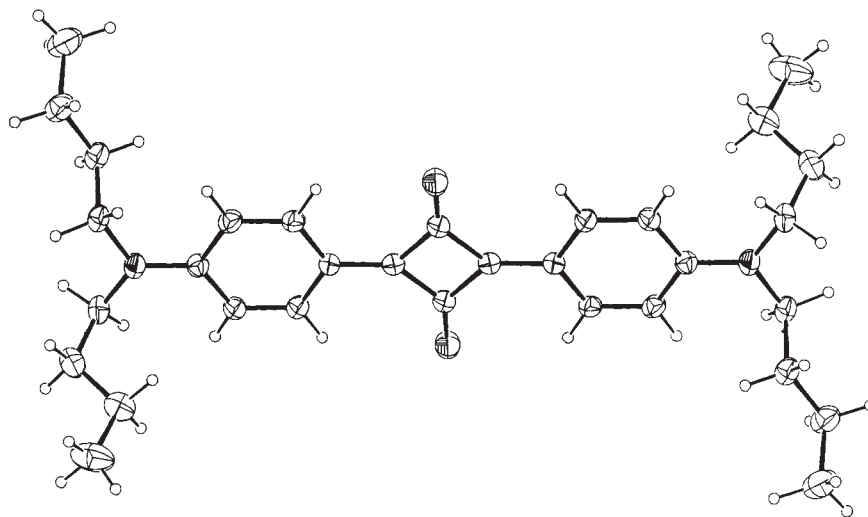
The X-ray crystal structure of a representative dye, 2,4-bis-(4-(*N,N*-dibutylamino)phenyl)squaraine, has confirmed that the chromophore is both planar and centrosymmetric (Fig. 1). The crystal has a centrosymmetric space group ($P2_1/c$ with

$a = 9.046$ (1), $b = 19.615$ (2), $c = 9.055$ (1) Å, $\beta = 116.07$ (5)°, $Z = 2$), and so the bulk material exhibits no SHG. In contrast, its LB films are SHG-active but are of poor quality whereas those of the 2,4-bis(4-(*N*-methyl-*N*-alkylamino)phenyl)squaraines show improved deposition characteristics and reproducible nonlinear optical properties (Table 1). Consequently, this Letter concerns *N*-methyl-*N*-alkylamino derivatives and, in particular, the hexyl homologue.

The compound 2,4-bis(4-(*N*-methyl-*N*-hexylamino)phenyl)squaraine was spread from chloroform solution onto the pure water subphase of an LB trough (model 622, Nima Technology, Coventry), left for 10 min at 25 °C and then compressed at 0.1 Å² per molecule per s. LB films were deposited on the upstroke by passing the substrate, either a glass slide for SHG measurements or a silver-coated slide for surface plasmon resonance studies, through the floating monolayer at 80 $\mu\text{m s}^{-1}$. The compression isotherm shows an area of ~ 67 Å² per molecule at zero pressure which decreases to 42 Å² per molecule at the onset of the plateau region (Fig. 2). This suggests that the chromophore initially resides with its long axis parallel to the air–water interface, and then gradually tilts upwards when compressed, the van der Waals dimensions of the chromophore being $17 \times 7 \times 3.5$ Å. But when deposited the orientation alters, resulting in a modified spectrum.

Molecular aggregation has a profound effect on the solid-state spectra of squaraine dyes and has been extensively studied for different crystal polymorphs^{9,10} and LB films^{11–14}. Law and Chen¹⁴ have reported that dipole–dipole interactions between the central groups of adjacent molecules result in an absorption maximum at ~ 530 nm, whereas intermolecular charge-transfer between the donor and acceptor parts results in a red-shifted band at ~ 650 nm. In our study, the floating monolayer exhibits both types of transition (Fig. 3a) and the spectrum mimics that of the purple triclinic phase of a congener reported by Tristani-Kendra and Eckhardt⁹. In contrast, the LB films show a single charge-transfer band whose maximum is red-shifted from 656 nm, when deposited at 5 mN m⁻¹, to 694 nm at 19 mN m⁻¹ or above (Fig. 3b). The spectrum resembles that of the blue-green monoclinic phase of the *N,N*-dibutylamino homologue

FIG. 1 Molecular structure of 2,4-bis(4-(*N,N*-dibutylamino)phenyl)squaraine showing the thermal ellipsoids. The chromophore is as shown in Fig. 2 but each of the alkyl groups is butyl.



(Fig. 1), and the observed shift may be attributed to a more favourable overlap of the donor and acceptor parts of adjacent molecules as the film is compressed.

Surface plasmon resonance studies were performed on glass-Ag-monolayer structures as described in ref. 15 and the reflectivities determined as a function of the angle of incidence using a frequency-doubled Nd:YAG laser ($\lambda = 532$ nm). The data shown in Fig. 4 were obtained for a film deposited at 19 mN m^{-1} and analysed by comparison with the Fresnel reflection formulae using a curve-fitting routine. The analysis yielded real (ϵ_r) and imaginary (ϵ_i) parts of the dielectric constant of 2.97 and 0.68, respectively, and a monolayer thickness (l) of $4.93 \text{ \AA}$. When compared with the van der Waals dimensions above, the thickness implies that the squaraine molecules pack parallel to the substrate with the chromophores tilted slightly out of the plane. Kim *et al.*¹⁶ have reported that the *N,N*-dipropylamino homologue packs in a similar manner when deposited onto glass substrates whereas other amphiphilic materials usually show vertical alignment. Thus, we believe that the horizontal packing of the squaraine is influenced by the short alkyl chain lengths.

The SHG from the monolayer films on glass was measured in transmission with the laser beam (Nd:YAG; $1.064 \mu\text{m}$) incident on the LB monolayer at 45° (see ref. 17). The polarization was rotated using a half-wave plate, the second-harmonic intensity being negligible for s-polarized light and moderately strong for p-polarized. The SHG is dependent on the deposition pressure. A more intense signal is obtained for films deposited at 19 mN m^{-1} and this is accompanied by an abrupt change in the aggrega-

tion spectrum (Fig. 3c). The optimum signal is comparable with the SHG from monolayer films of the more commonly reported donor-(π -bridge)-acceptor (D- π -A) dyes¹⁷⁻²² with an effective bulk susceptibility of 250 pm V^{-1} . However, as the susceptibility of the monolayer is proportional to the reciprocal of its thickness the values are enhanced by the fact that the molecules pack parallel to the plane of the substrate rather than on end. Nonetheless, the SHG is similar to that obtained from films of the hemicyanine halides¹⁷⁻²², and the spectral and nonlinear optical properties have remained unchanged for four months.

SHG has been observed in this study from several related squaraines (Table 1) and is unusual because the chromophore is centrosymmetric. As a result the non-zero susceptibilities cannot originate from intramolecular electric dipole contributions and also, the second-harmonic intensities are too high to be attributed to interfacial effects. Interestingly, weak SHG from films of buckminsterfullerene, C_{60} , has been attributed to magnetic dipole contributions¹⁻³, but as the susceptibility is very much smaller than observed here we believe that an alternative process must be responsible in our case. Chemical instability is firmly dismissed as the cause of the unusual behaviour; the solution spectra of the redissolved films are identical to those of the original crystals and we have found no evidence to suggest that the squaraine dyes are altered during the timescales of the LB deposition. Additionally, it should be noted that spun-coated films also exhibit SHG, albeit weaker due to the random orientation of the chromophores, and that the squaraine dye is stable in the solvent used.

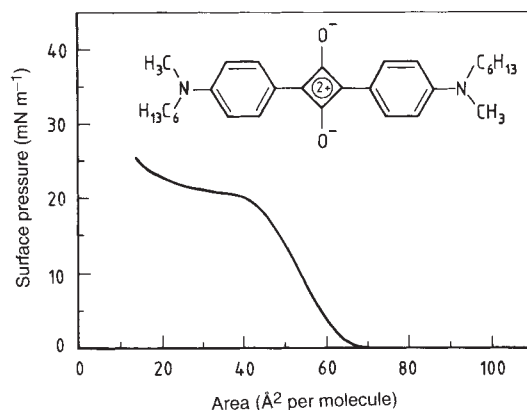


FIG. 2 Isotherm of 2,4-bis(4-(*N*-methyl-*N*-hexylamino)phenyl)squaraine obtained at 25°C and at a compression rate of $0.1 \text{ \AA}^2$ per molecule per s. Inset; molecular structure.

TABLE 1 Nonlinear optical properties and spectra of monolayer films of the 2,4-bis(4-(*N*-methyl-*N*-alkylamino)phenyl)squaraine dyes

Alkyl group	Deposition range (mN m^{-1})	SHG*	λ_{max} (nm)
Butyl	5–20	$>3^\dagger$	675–687
Hexyl	5–19	0.2–1.1	656–694
Octyl	10–20	0.6–0.7	681–686
Decyl	10–20	0.4–0.5	662–687
Dodecyl	10–20	0.1–0.2	669–671
Tetradecyl	10–20	0.2–0.3	669–677
Hexadecyl	10–30	0.1–0.2	667–670
Octadecyl	10–30	0.4–0.6	670–673
Eicosyl	10–30	0.4–0.5	669–677
Docosyl	10–30	0.2–0.3	670–672

* All values relative to the mean SHG from LB monolayers of the hemicyanine dye, *E-N*-docosyl-4-[2-(4-dimethylaminophenyl)ethenyl]pyridinium bromide.

[†] The SHG from films of the butyl homologue rapidly reduced after deposition whereas that of the hexyl homologue remained unchanged for more than four months.

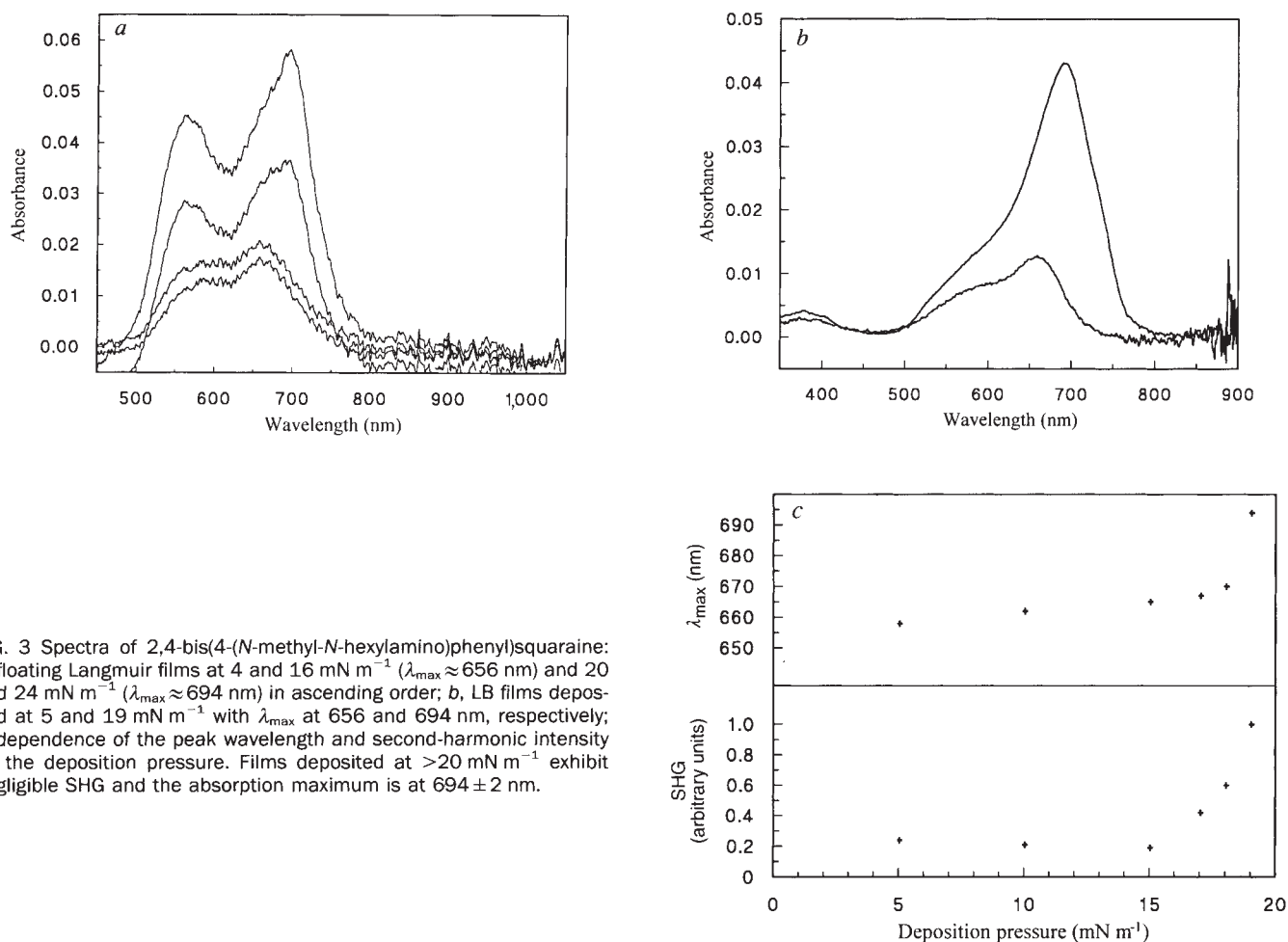


FIG. 3 Spectra of 2,4-bis(4-(*N*-methyl-*N*-hexylamino)phenyl)squaraine: *a*, floating Langmuir films at 4 and 16 mN m⁻¹ ($\lambda_{\text{max}} \approx 656$ nm) and 20 and 24 mN m⁻¹ ($\lambda_{\text{max}} \approx 694$ nm) in ascending order; *b*, LB films deposited at 5 and 19 mN m⁻¹ with λ_{max} at 656 and 694 nm, respectively; *c*, dependence of the peak wavelength and second-harmonic intensity on the deposition pressure. Films deposited at >20 mN m⁻¹ exhibit negligible SHG and the absorption maximum is at 694 ± 2 nm.

For conventional D- π -A materials the intramolecular charge-transfer contribution to the molecular hyperpolarizability is larger than the sum of all other effects²³ and thus, for the symmetrical squaraines, the strong SHG can be explained (in part) if we assume that there is a significant intermolecular charge-transfer contribution. Based on the assignment of Law and Chen¹⁴, the LB film spectra indicate intermolecular charge-trans-

fer between the donor (dialkylamino) and acceptor (four-membered ring) groups. However, to satisfy the structural criteria for SHG we must postulate that the donor-acceptor-donor (D-A-D) molecules which comprise the dimer, or higher aggregate, adopt a non-parallel arrangement with a single close contact between the donor and acceptor groups of a molecular pair. Interestingly, the X-ray crystal structure of the *N,N*-dibutylamino homologue (manuscript in preparation) shows a parallel alignment of molecules inclined to the *ac* plane and a herringbone-like arrangement parallel to the *b* axis; the structure is centrosymmetric, space group *P2₁/c*, whereas each of the charge-transfer "dimers" which comprise the herringbone is acentric. Thus, the structural criteria for SHG may be satisfied if the herringbone arrangement is maintained within the LB film and if the repeating "dimer" motif does not pack centrosymmetrically.

Regardless of the precise mechanism, the results given in Table 1 show that materials exhibiting strong SHG can be obtained from centrosymmetric molecules. □

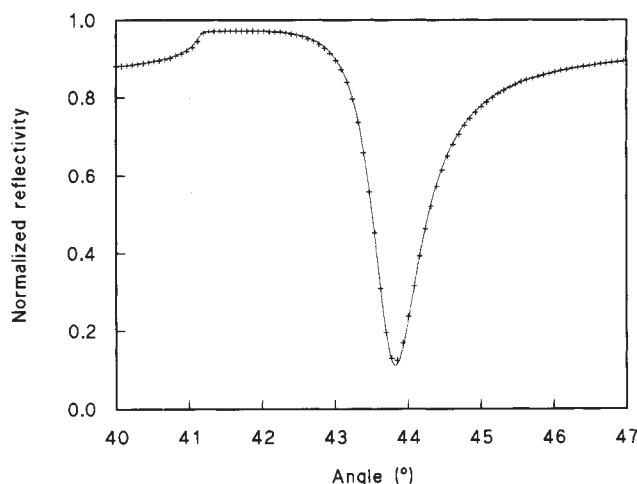


FIG. 4 Normalized reflectivity from a glass-Ag-monolayer structure of the 2,4-bis(4-(*N*-methyl-*N*-hexylamino)phenyl)squaraine dye deposited onto a 44 nm thick overlay of silver at 19 mN m⁻¹. The solid line corresponds to the theoretical fit with $\epsilon_s = 2.97$, $\epsilon_i = 0.68$ and $l = 4.93$ Å.

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Transition-state stabilization as a measure of the efficiency of antibody catalysis

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THERE are now about 60 examples of reactions that have been successfully catalysed by monoclonal antibodies^{1–3}. Not surprisingly, many of the early examples involved reactions that were already favoured kinetically (such as carbonate and ester hydrolysis). But it has since been shown that antibodies can also accelerate reaction pathways that are normally disfavoured kinetically (by at least a few kcal mol⁻¹)^{4–7}. Here we use transition-state theory to provide a quantitative analysis of the scope and limitations of antibody catalysis. We show that the observed rate accelerations can be predicted from the ratio of equilibrium binding constants of the reaction substrate and the transition-state analogue used to raise the antibody. This scheme allows us to rationalize the product selectivity displayed in antibody catalysis of disfavoured reactions, to predict the degree of rate acceleration that catalytic antibodies may ultimately afford, and to highlight some differences between the way that they and enzymes catalyse reactions.

The relationship between the catalytic activity of an antibody and the differential binding of the transition state relative to the substrate can be most clearly visualized by use of a thermodynamic cycle based on transition-state theory (Fig. 1). Because such an analysis has previously been performed by others for both enzyme-⁸ and antibody-catalysed reactions^{9,10}, we omit the details of the derivation. The important relation is that $K_M/K_i \approx k_{cat}/k_{uncat}$ for unimolecular reactions; an analogous treatment of a random bimolecular mechanism yields $(K_{M,1}K_{M,2})/K_i \approx k_{cat}/k_{uncat}$. Here K_M is the equilibrium constant for antibody binding of the reaction substrate, and K_i is that for binding of the reaction transition state, which can be approximated by the equilibrium constant for binding of the hapten. As demonstrated below, these relationships are obeyed in most cases. Thus, to the extent that the transition-state analogue used to induce the antibodies accurately mimics the true transition state of the reaction, the key to consistently isolating antibodies with high catalytic efficiencies (that is, those with $k_{cat}/k_{uncat} > 10^4$) is to maximize the chances of discovering those

antibodies that possess high affinity for the transition-state analogue, either by rational engineering^{11,12}, or by selections from combinatorial libraries of antibody genes (reviewed in ref. 13).

Although the data shown in Fig. 2 generally conform to the above relationships, several significant issues complicate our analysis. The first, errors in the measurement of K_i , often arise when data are incorrectly analysed using equations derived for the condition that the concentration of the transition state is very much greater than that of the antibody ($[I] \gg [Ab]$). Deviations, in some cases deliberate, between the structure of the transition-state mimic and the true transition state are another reason that the above relationship is not obeyed precisely. (For example, the phosphonic acid derivatives commonly used to mimic the tetrahedral intermediates encountered in hydrolysis reactions of acyl derivatives are far more acidic than their carbon counterparts.) Our analysis is also limited by the assumption that the antibody-catalysed reaction proceeds through a single transition state in which the chemical step is cleanly rate-limiting (as it is this step that is mimicked by the transition-state analogue). While such a supposition would clearly be invalid for many enzymes, in which evolution has 'shaped' the enzyme to facilitate several steps in the chemical transformation, the absence of evolutionary pressure on antibody catalysts makes this assumption more reasonable in the present case. Finally, we presume that the antibody-catalysed reaction and its uncatalysed counterpart proceed by substantially the same mechanism. A breakdown of this assumption generally manifests itself as a negative deviation from the correlation described in Fig. 2, that is, the antibody has less catalytic activity than predicted⁸. We expect this complication to be relatively uncommon for reactions catalysed by antibodies, because the choice of hapten programmes the actual reaction mechanism.

Despite these caveats, a striking feature of the thermodynamic analysis is its independence of reaction type. The data used to construct the two plots were drawn from catalytic antibodies (abzymes) that catalyse hydrolyses, pericyclic reactions, decarboxylations, eliminations, a porphyrin metallation as well as amide and ester synthesis. Interestingly, the thermodynamic relationship correctly predicts the catalytic activities of antibodies raised against antigens that were designed to elicit specific functional groups at appropriate distances (the bait-and-switch strategy), rather than mimic the transition state. Although this may be a fortuitous coincidence, we also note that in the three examples included, the antigens still generally resemble the high-energy intermediates that would occur during catalysis. Some of the deviations from this relationship are also illuminating. In the case of an antibody raised against a *p*-nitrophenyl phosphonamide that catalyses the hydrolysis of an activated amide and a series of esters¹⁴ the acylation rates for the *p*-nitrophenyl amide and ester fall very close to the theoretical line; however, as the *para*-substituent is varied (Cl, CHO, COCH₃, CH₃), the points

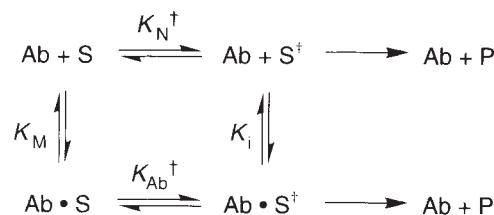


FIG. 1 Thermodynamic cycle comparing the rate of a unimolecular antibody-catalysed reaction with its uncatalysed counterpart⁸. In this cycle, based on transition-state theory, the affinity of an antibody (Ab) for the transition state ([‡]) of the reaction is approximated by the K_i value for the inducing antigen. K_M and $K_{Ab}^\ddagger$ represent the equilibrium constants between the ground and transition states of the antibody-catalysed and uncatalysed reactions, respectively. S, substrate; P, product.

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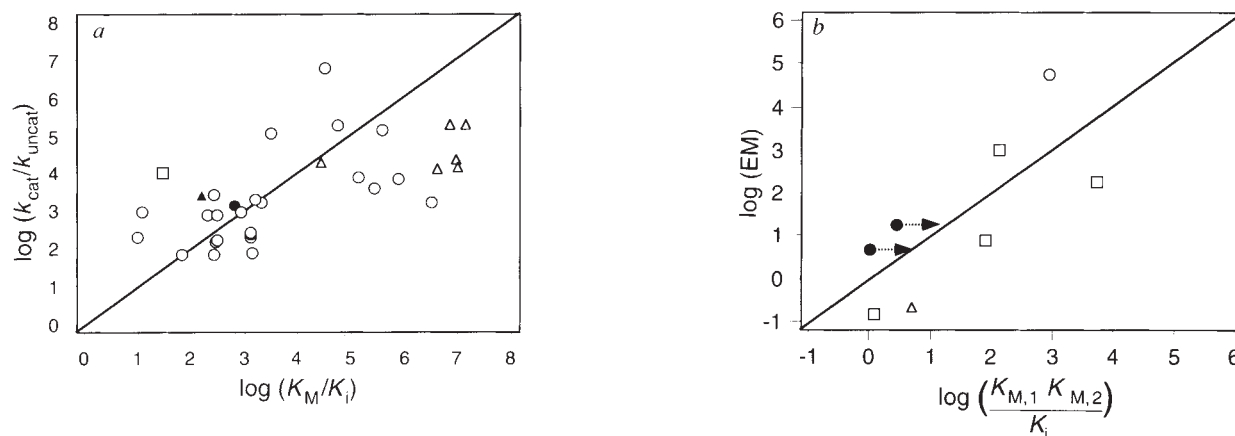


FIG. 2 *a*, Comparison of available binding energy (defined as K_M/K_i) with observed catalytic activity (defined as k_{cat}/k_{uncat}) for antibody-catalysed unimolecular reactions. The line (which has a slope of +1) is not a fit to the data; it is included only for reference. The data are grouped by reaction type: ○, hydrolysis; □, Claisen rearrangement; △, decarboxylation; ●, elimination; ▲, porphyrin metallation. In one case, the data represents a lower limit as a result of uncertainty in the value of K_i (indicated by a dashed arrow). Kinetic data have been extracted from refs 19–36 and a complete listing of the data is available (see Supplementary Information). Five cases show pronounced positive deviations from our correlation. Four of these are acylation or dephosphorylation reactions that are particularly prone to contaminating enzymic activities and which should therefore be viewed with caution. The origin of the

deviation in the case of the Claisen rearrangement²⁰ is deserving of further investigations. *b*, Comparison of available binding energy (defined as $(K_{M,1}K_{M,2})/K_i$) with effective molarity (EM, defined as k_{cat}/k_{uncat}) for antibody-catalysed bimolecular reactions. The line (which has a slope of +1) is included for reference. The data are grouped by reaction type: ○, ester synthesis; □, amide synthesis; △, imine formation; ●, Diels-Alder reaction. In two cases, the data represent lower limits (indicated by dashed arrows). Kinetic data have been extracted from refs 7, 9, 37–39 and a complete listing of the data is available (see Supplementary Information). Only those antibodies for which $K_i \leq 1 \times 10^{-6}$ M are shown (values of $K_i \geq 1 \times 10^{-6}$ M are more likely to reflect active site titration rather than true measures of K_i).

deviate progressively further in the negative direction. We suggest that such behaviour reflects differences in interactions between the antibody and the transition states for these substrates (particularly with the *para* substituent), so that K_M/K_i no longer represents a true measure of the available transition-state binding energy. On balance, the success of the correlation suggests that transition-state stabilization primarily defines the efficiency of antibody catalysis.

The above exercise was concerned primarily with understanding the rate enhancements observed for antibody catalysis of favourable reactions from an analysis of the transition-state binding energies. However, its success in the majority of cases also allows one to look at these energetic relationships from another perspective: given a total differential binding energy defined by K_M/K_i , can an antibody accelerate a normally unobservable reaction pathway? Before considering this question in detail, we note that there are two general means by which antibodies might catalyse disfavoured reactions that differ in the locations of the rate- and product-determining steps. For example, an antibody could first catalyse the rate-determining formation of a high-energy species with the potential to yield numerous products, then direct its exothermic decomposition to a single product by controlling access to a specific reactive conformation. This strategy is exemplified by the antibody-catalysed cationic cyclization of olefin **1** that affords almost exclusively cyclohexanol **3** (Fig. 3a), rather than the spectrum of products formed in the uncatalysed counterpart⁴. Alternatively, an antibody might alter the relative heights of the individual transition-state barriers that connect the substrate's ground state to the various products, thereby favouring one reaction pathway over another. Although the first approach is inherently less demanding than the second, both are founded on the use of binding energy for catalysis. Below, we consider four examples of abzymes that utilize the second strategy and find that in each case, the available binding energy is sufficient to selectively direct formation of the normally unfavourable product.

The elimination of fluoride from **4** to form *Z*-olefin **5** occurs via the normally disfavoured *syn*-elimination pathway (Fig. 3b). The recent report⁵ of selective activation of this *syn*-elimination

pathway can be rationalized in terms of the differential binding of the transition state relative to the substrate (K_M/K_i) which we estimate as ~ 6 kcal mol⁻¹, in accord with recent calculations that place the transition state for *syn* elimination ~ 5 kcal mol⁻¹ higher in energy than the corresponding one for *anti* elimination⁵. [Our estimate of 6 kcal mol⁻¹ is obtained as follows. Based on kinetic simulations of the published data, the antigen K_i value was ~ 10 nM. From this, $K_M/K_i \approx 2 \times 10^4$ and the available transition-state binding energy is thus ~ 6 kcal mol⁻¹. To estimate the value for k_{uncat} , we assume that the uncatalysed rate of *syn* elimination could be no greater than 1% of that for the *anti* pathway (because the *syn*-product was undetectable), so that $k_{uncat} \leq 2.5 \times 10^{-6}$ min⁻¹. From this, $k_{cat}/k_{uncat} \geq 60$.] Given the nature of the inducing antigen (a substituted [2.1.2]-bicycloheptane), it is intriguing to speculate that the antibody selectively binds the small population of **1** whose conformation is already close to the necessary *syn* arrangement, rather than catalysing bond rotation before elimination. [The value of k_{cat}/K_M for this antibody (1.2×10^{-2} M⁻¹ s⁻¹) is 10 orders of magnitude lower than the rate of a diffusion-controlled process, consistent with this suggestion.] A similar analysis can be applied to recent reports of antibody-catalysed ring closure, cycloadditions and peptide synthesis. The 6-*endo*-tet pathway for ring closure of hydroxy epoxide **6** to pyran **8**, rather than the normally observed 5-*exo*-tet mode of addition to furan **7** (Fig. 3c), is estimated to proceed through a transition state ~ 1.9 kcal mol⁻¹ higher in energy⁶. In the case of the antibody-catalysed reaction, we estimate from K_M/K_i that $\Delta\Delta G^\ddagger \geq 3.5$ kcal mol⁻¹ (where $\Delta\Delta G^\ddagger$ is the available transition-state binding energy for the substrate *G*), providing sufficient binding energy to favour the formation of the 6-*endo*-tet product **8**. [We estimated the value of K_M/K_i as ~ 500 , based on the observation that addition of a stoichiometric amount of antigen completely inhibited catalysis.] In general, uncatalysed Diels-Alder cycloadditions exhibit large preferences for *endo*-products, with differences in transition-state energies of ~ 2 kcal mol⁻¹. However, this stereochemical outcome has recently been altered by antibody catalysts that exclusively produce either the *endo* product **11** or *exo* product **12** from the reactants **9** and **10** (Fig.

3d)⁷. We estimate K_i values of ~ 70 nM for both antigens and calculate the differential binding affinities (defined as $(K_{M,1} \times K_{M,2})/K_i$) as ~ 2.6 and 2.0 kcal mol⁻¹ for the *exo*- and *endo* products, respectively. This stabilization is sufficient to direct the formation of the desired product in ratios $>99:1$. To take a final example, the antibody-catalysed formation of dipeptide **15** from the activated ester **13** and acceptor amine **14** seems to involve a tetrahedral species in which the acyl donor partitions almost exclusively to amide formation rather than hydrolysis (Fig. 3e)¹⁵. The capability to use water selectively as a reagent or to block its access to a reactive species is a common feature of enzymic catalysis, now successfully recapitulated with abzymes.

From this analysis, it is clear that one of the promising aspects of catalytic antibodies will be to direct a reaction down a particular path within a complex product manifold, as free-energy differences of 2–3 kcal mol⁻¹ are sufficient for the dominance of a single product ($>98\%$). Interaction energies of up to 5.5 kcal mol⁻¹ are generally accessible ($K_M/K_i \approx 1 \times 10^4$), although in favourable cases K_M/K_i can reach 1×10^7 , affording 9.6 kcal mol⁻¹ in interaction energy (Fig. 1). At present, abzymes with k_{cat}/k_{uncat} values of $\sim 10^6$ seem to represent an upper limit on turnover ($K_M/K_i = 10^{-4}$ M/ 10^{-10} M), given the tighter affinities observed for some haptens and a value for substrate binding sufficient for adequate flux at millimolar substrate concentrations. Alternatively, this interaction energy can be applied to overcoming the barrier to a disfavoured reaction. In either case, K_i emerges as a major determinant of the rate constant for antibody turnover.

How can we use this information to improve the catalytic efficiency of abzymes? We see two interrelated problems that

must be addressed. First, the affinity of antibodies for their respective transition states must be improved. In the case of enzymes, such affinities can reach values as high as 10^{-24} M, far exceeding the 10^{-9} M values observed for the best abzymes and this is reflected in their rate accelerations of $>10^{17}$ over background¹⁶. The second problem concerns the dynamics of the antibody/transition state interaction. Catalytic antibodies are elicited to a single, specific structure; by contrast, enzymes have evolved to recognize a series of structures that connect the substrate and product along the reaction coordinate. Consequently, the binding energy is applied to preferential transition-state stabilization by abzymes whereas enzymes may also act to destabilize bound substrates.

The difference between abzymes and enzymes is also manifested in their interactions with transition-state analogue inhibitors. In the case of enzymes, binding of transition-state analogues almost universally involves a slow isomerization of the initial encounter complex to the tight-binding form⁸. This observation is difficult to reconcile with the notion that enzymes have evolved to present a rigid surface with maximal complementarity to the transition state of a reaction, but instead suggests that enzymes present a flexible surface that stabilizes not only the transition state, but which also informs the intermediate states along the reaction trajectory. Although antibodies can also exhibit structural flexibility in their binding interactions¹⁷, for the one catalytic antibody that has been examined in detail, the binding of the transition-state analogue seemed to be a simple, one-step reaction¹⁸. It therefore seems likely that the allowed structural dynamics of antibodies may not have been optimized for catalysis, and we speculate that this may explain some of the negative deviations from our correlation (that is,

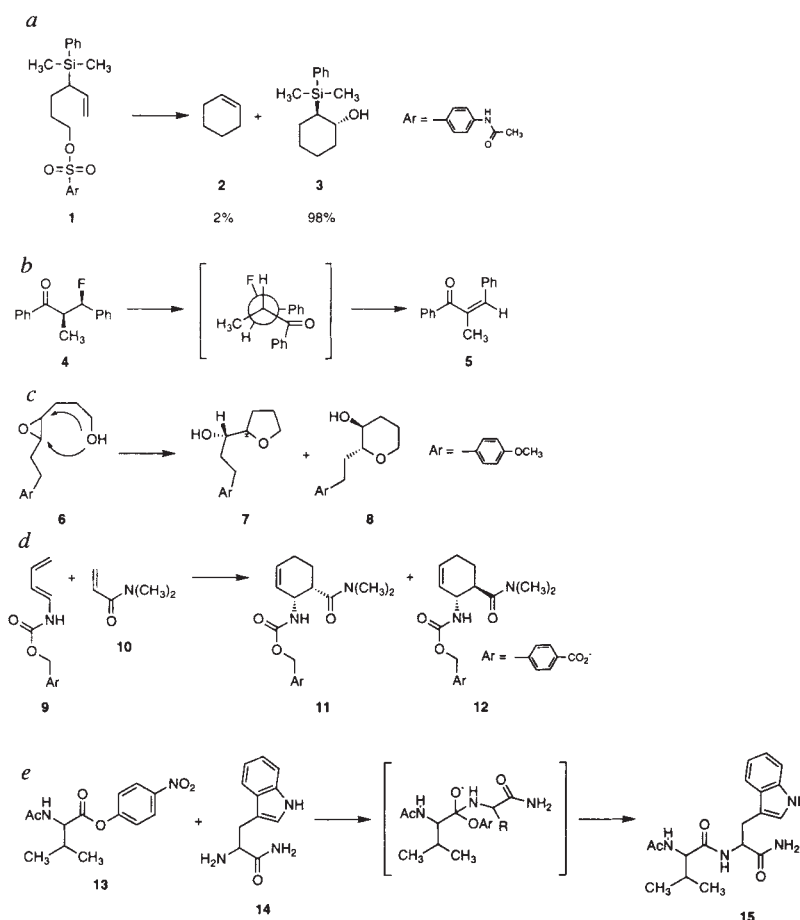


FIG. 3 Abzyme-catalysed reactions that yield normally disfavoured products. *a*, Cationic olefin cyclization; *b*, *syn*-elimination; *c*, selective 6-endo-tet epoxide opening; *d*, Diels-Alder cycloaddition; *e*, peptide bond formation.

those antibodies whose catalytic efficiency is less than that predicted on the basis of the available binding energy). Provided that our conjecture is correct about the structural limitations imposed on the catalytic antibody by the very nature of its induction, methods that focus on altering not only the identities but also the presentation of catalytic residues may offer a powerful means of evolving such initial catalysts to higher activities; in so doing, we will learn more about enzymes. □

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Possible role of climate in the collapse of Classic Maya civilization

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THE Maya civilization developed around 3,000 years ago in Mesoamerica, and after flourishing during the so-called Classic period, it collapsed around 750–900 AD¹. It has been speculated^{2–6} that climate change may have played a part in this collapse. But efforts to reconstruct the last three millennia of Mesoamerican climate using palynological methods have met with equivocal success, because human-mediated deforestation has altered regional vegetation in ways that mimic climate shifts, making it difficult to discriminate between natural and anthropogenic changes^{7–15}. Here we use temporal variations in oxygen isotope and sediment composition in a 4.9-m sediment core from Lake Chichancanab, Mexico, to reconstruct a continuous record of Holocene climate change for the central Yucatan peninsula. The interval between 1,300 and 1,100 yr BP (AD 800–1,000) was the driest of the middle to late Holocene epoch, and coincided with the collapse of Classic Maya civilization. This continuous climate proxy record thus provides evidence of climate deterioration in the Maya region during the terminal Classic period.

We investigated the sediment record of Lake Chichancanab (Fig. 1), the largest closed-basin lake in Yucatan, Mexico¹⁶. 'Chichancanab' means 'little sea' in Yucatec Maya, which is an appropriate name in view of the lake's sulphate-rich (2,545 mg l⁻¹), high-salinity waters (total dissolved solids,

4,000 mg l⁻¹). Today, lake water is at or near saturation with respect to both calcite (CaCO₃) and gypsum (CaSO₄). Although gypsum does not precipitate in the open lake today, gypsum crystals are found in littoral areas and at depth in sediments. Past variations in the saturation state of Lake Chichancanab's water with respect to calcite and gypsum are reflected by changes in the relative proportion of these minerals in the sediment. We assume that downcore shifts in the relative abundance of calcite and gypsum in sediments reflect changes in the hydrologic budget of the lake that were ultimately controlled by changes in the ratio between evaporation and precipitation (E/P).

Regional climate today is characterized by a mean annual temperature of 25.4 °C and yearly rainfall of 1,300 mm, with a rainy season extending from May to September and a dry season from October to April^{16,17}. Lake evaporation during the dry season is considerable, and controls the oxygen isotopic composition of Lake Chichancanab's waters. The average oxygen-isotope composition ($\delta^{18}\text{O}$) of lake water in 1993 was 3.24‰, which is slightly less than the range of 3.4 to 5.4‰ reported in 1973¹⁶. The lake water is isotopically heavier than the long-term average $\delta^{18}\text{O}$ of precipitation for the region (weighted mean, -4.0‰)¹⁸. In 1973, $\delta^{18}\text{O}$ values of rain and spring water at Chichancanab were -6.1‰ and -4.7‰, respectively¹⁶. H₂¹⁸O enrichment in lake water relative to precipitation and ground water reflects evaporative loss of H₂¹⁶O from the lake¹⁶. Past changes in the ¹⁸O/¹⁶O ratio of lake water are recorded in the $\delta^{18}\text{O}$ of carbonate in gastropod and ostracod shells preserved in lake sediments.

We used the oxygen-isotope signal in shell carbonate and the gypsum/calcite ratio in the sediments to reconstruct past changes in E/P. Our working assumption is that times of high E/P (that is, dry climate) are reflected by increased $\delta^{18}\text{O}$ values of shell carbonate and an increased proportion of gypsum to calcite in sediments. Conversely, periods of low E/P (that is, wetter climate) are marked by low $\delta^{18}\text{O}$ values and a decreasing proportion of gypsum to calcite.

Near the base of the core, earlier than 8,200 yr before present (BP), a terrestrial environment is indicated by the presence of

land snails (for example, *Gastrocopta* (Pupillidae) and *Cecilioides*; F. Thompson, personal communication) (Fig. 2). Covich¹⁹ also reported the occurrence of terrestrial gastropods before 8,000 yr BP in a core from the southern basin of Lake Chichancanab (that is, Lake Esmeralda, Fig. 1). Both basins were dry during the late Glacial and earliest Holocene, consistent with evidence for lowered water tables and aridity at many sites in the lowland neotropics^{20–28}. At 8,200 yr BP, filling of the lake basin is marked by the onset of gypsum precipitation (Fig. 2). A 7-cm-thick charcoal layer occurs just above the increase in sulphur concentration, and is dated directly at $7,560 \pm 35$ yr BP or between 7,920 and 7,790 yr BP using the 'terrestrial' regression equation (Table 1). The relatively high $\delta^{13}\text{C}$ of the charcoal (mean, -14.4‰) indicates that it was derived from C_4 vegetation. Microscopic examination of carbonized stem and root fragments suggests remains of burned grasses (L. Newsom, personal communication). Increases in charcoal abundance near the Pleistocene/Holocene boundary have been reported elsewhere in Central America, and are attributed to either natural or human-induced fires^{27–30}.

Immediately above the charcoal layer, from 7,750 to 7,260 yr BP, sediments contain abundant, well-preserved tests of the benthic foraminifer *Ammonia beccarii* (Fig. 2). Covich¹⁹ also reported *A. beccarii* at 400 cm in a core from the southern basin of Chichancanab. Although most species of benthic foraminifera are exclusively marine, *A. beccarii* tolerates a wide range of temperatures (10–35 °C) and salinities (7–67 parts per thousand, p.p.t.), but reproduces only at salinities between 13 and 40 p.p.t. (ref. 31). The presence of *A. beccarii* between 7,750 and 7,260 yr BP indicates that the salinity of Chichancanab was higher than today's value of 4 p.p.t. Furthermore, $\delta^{18}\text{O}$ values of ostracods and gastropods are enriched between 7,800 to 7,200 yr BP, indicating high E/P conditions (Fig. 2).

Lake filling at ~8,200 yr BP was related to sea level rise during the last deglaciation. As sea level rose, so did the level of the freshwater aquifer in Yucatan^{16,19}. The filling of Lake Chichancanab occurred during the latter part of the second stage of deglaciation that began at 10,000 yr BP³² and lasted until 7,000–6,000 yr BP. The rapid rise in lake level occurred ~7,200 yr BP, when calcite precipitation replaced gypsum deposition and $\delta^{18}\text{O}$ values of ostracods and gastropods decreased abruptly (Fig. 2). Sea level continued to rise slowly after 6,000 yr BP, but the rate was insufficient to substantially affect lake level during the Middle to Late Holocene.

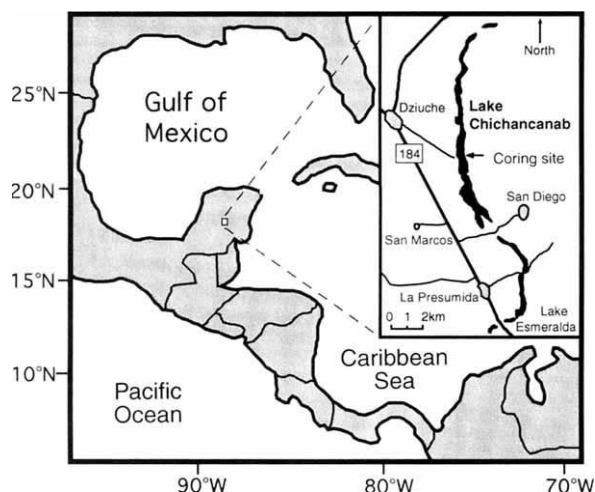


FIG. 1 Map of Central America; inset shows the location of Lake Chichancanab ($19^{\circ} 50' \text{ N}$, $88^{\circ} 45' \text{ W}$, elevation ~15 m above m.s.l.), Yucatan, Mexico. A 4.9-m core was retrieved in 6.9 m of water from the central basin of Chichancanab using a square-rod piston corer and sediment-water interface corer. The core was dated by radiocarbon measurements on charcoal, seed and shell material (Table 1).

TABLE 1 Age analysis of sediment core from Lake Chichancanab

Sample type	Depth (cm)	Accession* number	Age (yr)	Error ($\pm$ yr)
Terrestrial:				
Seed	65	OS-3545	1,140	35
Charcoal	421	OS-2157	7,560	35
Charcoal	421	CAMS-12780	7,600	60
Charcoal	421	CAMS-12781	7,460	60
Land snail	472	OS-2052	9,180	50
Aquatic:				
<i>Pyrgophorus</i>	15	CAMS-12900	1,550	60
<i>Pyrgophorus</i>	65	OS-3443	1,600	30
<i>Pyrgophorus</i>	103	OS-3446	3,200	40
Bivalves	142	OS-2148	5,210	30
<i>Pyrgophorus</i>	238	OS-3445	7,100	30
<i>Pyrgophorus</i>	314	OS-3444	9,040	65
Mixed gastropods	350	OS-2051	8,680	45
Mixed gastropods	385	OS-2729	9,530	60
Bivalves	406	OS-2055	9,500	50

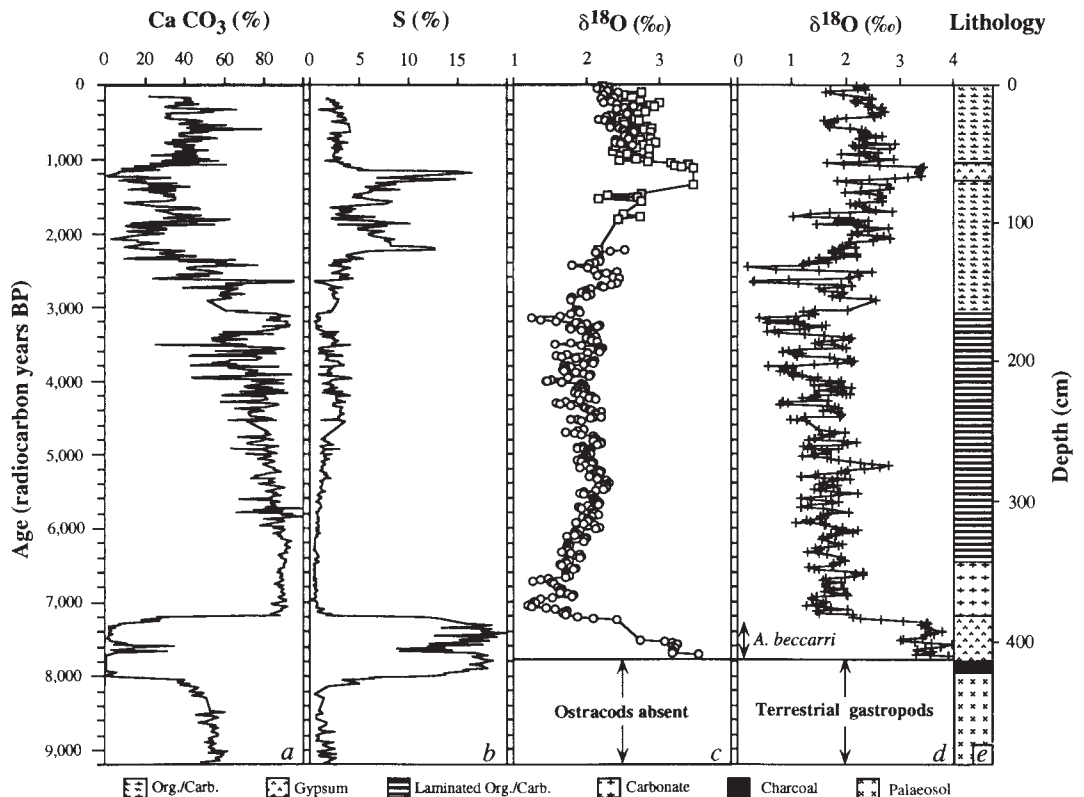
Radiocarbon analyses were performed on both 'aquatic' shells and 'terrestrial' material (seed, charcoal, land snail) from the Chichancanab core. Because of Lake Chichancanab's hard water, radiocarbon dates on 'aquatic' shells are artificially old (that is, older than their true radiocarbon age) because shells incorporate ^{14}C -deficient carbon derived from dissolution of local limestone. In contrast, 'terrestrial' materials are immune to the vagaries of hard-water-lake error because they derive their carbon from atmospheric CO_2 . The age–depth regression equations for aquatic and terrestrial materials are: aquatic, age = $22.271(\text{depth}) + 1,163$ ($r = 0.979$); terrestrial, age = $18.921(\text{depth}) - 62$ ($r = 0.998$). These regression lines have nearly the same slope and indicate a linear sedimentation rate of $\sim 0.5 \text{ mm yr}^{-1}$. The y-intercept for the 'aquatic' regression line suggests a modern hard-water-lake error of $\sim 1,200$ yr. Depths in the core were converted to age using the 'terrestrial' age–depth regression equation that is not affected by hard-water-lake error.

Relatively wet conditions (low E/P) between 7,100 and ~3,000 yr BP are indicated by high calcite and low sulphur contents of the sediment, and relatively low $\delta^{18}\text{O}$ values (Fig. 2). This interval coincides with the Early to Middle Holocene moist period that has been documented elsewhere in the neotropics^{16,21,24,29,33,34}. Hodell *et al.*²⁴ suggested that increased neotropical precipitation during the Early to Middle Holocene was caused by increased intensity of the annual cycle, a consequence of changes in seasonal insolation forced by the Earth's precessional cycle. The inferred climate history from Lake Chichancanab is remarkably similar to that from Lake Miragoane, Haiti, which indicates low lake salinity and moist conditions from ~7,000 to 4,000 yr BP^{24,35}.

During the Late Holocene, climate around the Caribbean Sea became drier relative to the wetter period that prevailed during the Early to Middle Holocene. About 3,000 yr BP, carbonate content of the sediments began to decrease and $\delta^{18}\text{O}$ values of ostracods started to increase, suggesting the inception of a drying trend (Fig. 2). The interval from 2,200 to 1,200 yr BP was marked by relatively high sulphur content and increasing $\delta^{18}\text{O}$ values, indicating distinctly drier conditions (Fig. 2). A similar period of exceptionally dry climate was reported from Lake Miragoane between 2,500 and 1,500 yr BP^{24,35}. The dry period at Lake Miragoane apparently occurred 300 yr earlier than at Chichancanab, but the difference in timing may be attributed to underestimation of the magnitude of hard-water-lake dating error in Lake Miragoane³⁵.

The driest climate conditions occur between ~1,300 and 1,100 yr BP, reaching a maximum value at $1,140 \pm 35$ yr BP (Fig. 2). The dating of peak aridity is based on radiocarbon analysis of a seed from 65 cm depth in the core (Table 1). Radiocarbon ages were converted to calendar years using the programme CALIB³⁶ and the decadal tree-ring dataset³⁷, yielding an age range of ~AD 800–1,000 for the arid period with the peak occur-

FIG. 2 The core from Lake Chichancanab was sampled at 1-cm intervals for measurement of CaCO_3 content (a), total sulphur content (b) and $\delta^{18}\text{O}$ values of ostracods (c) and the gastropod *Pyrgophorus coronatus* (d). (The $\delta^{18}\text{O}$ values are w.r.t. the PDB standard.) Oxygen-isotope data were smoothed using a three-point running mean. Because of variable abundance and preservation of ostracods, it was necessary to measure two species for stable isotopes. Circles represent measurements of *Cypria ophthalmica* and squares represent *Cyprinotus cf. salinus*⁴¹. The overlap of these species near the top of the core indicates little oxygen-isotope offset between species. The stratigraphic range of the benthic foraminifer *Ammonia beccarii* is shown in d. This zone is underlain by a 7-cm-thick charcoal horizon, and sediments at and below this level are devoid of ostracods and aquatic gastropods indicating a terrestrial environment. Column e shows the general lithology of the section. Two periods of particularly dry climate (high E/P) are marked by coincident peaks of sulphur content (gypsum) and $\delta^{18}\text{O}$ values of



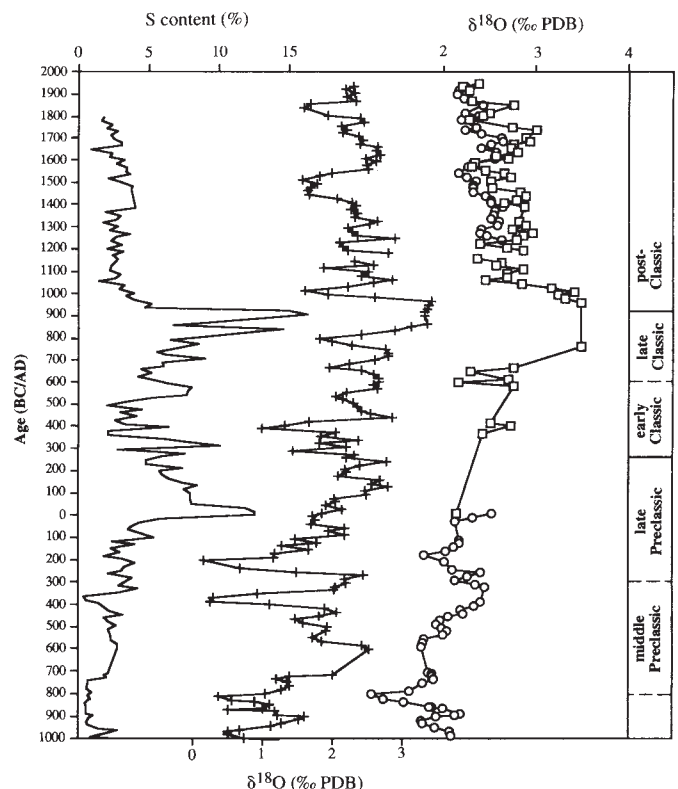
both ostracods and gastropods. The first occurs between 8,000 and 7,200 yr BP during the Early Holocene, and the second is centred at 1,200 yr BP during the Late Holocene.

ring at AD 922 (Fig. 3). The timing of this climate drying corresponds approximately to the collapse of the Classic Maya civilization between AD 750 and 900¹.

Although others have suggested that drought may have played a role in the Classic Maya collapse²⁻⁶, results from Lake Chichancanab provide the first unambiguous evidence for climate drying between AD 800 and 1000. The continuous Holocene record from Chichancanab sets this event within a long-term climate context. Aside from the period between 8,000 and 7,200 yr BP, when the lake was filling, aridity between 1,300 and 1,100 yr BP (AD 800–1000) represents the driest episode of the past 8,000 yr (Fig. 2).

The palaeoclimate record from Lake Chichancanab alone cannot support the case for regional late Classic drying, but reported low lake stands in central Mexico between 1400 and 900 yr BP³⁸ and increased fires in Costa Rica³⁹ may mark the same climate shift⁴⁰. Although the sediment record from Chichancanab suggests a causal link between climate and the late Classic collapse, the region inhabited by the Maya was geologically, ecologically and climatically diverse. The response of climate to any single forcing factor may not have been the same throughout the Mayan region, and so the pattern of cultural change may not

FIG. 3 Sulphur content and three-point running average of $\delta^{18}\text{O}$ values plotted versus calendar years to 1000 bc. Proxy signals of palaeoclimate (E/P) are compared with major subdivisions of Maya cultural evolution. Symbols are the same as in Fig. 2. The peaks in total sulphur content and $\delta^{18}\text{O}$ values are centred at ~AD 900 and indicate increased aridity during the terminal Classic period.



have been uniform. For example, the collapse seems to have been strongest in the southern Maya lowlands with populations shifting to the north¹. Even if the drying were regional in extent, however, the ecological and anthropological effects could have varied spatially, depending on the magnitude of subregional climate change and the sensitivity of the natural and cultural systems to environmental change. Further study is needed to determine the full magnitude and geographical extent of the dry period that occurred between AD 800 and 1000, and to explain the intraregional pattern of the collapse of Classic Maya civilization. □

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Young peridotitic diamonds from the Mir kimberlite pipe

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MINERAL inclusions in diamonds contained in the explosive volcanic rocks known as kimberlites provide an important record of geochemical processes in the continental lithosphere. Samarium-neodymium model ages as old as 3.2 Gyr have been obtained for many peridotitic mineral inclusions^{1–4}, pointing to the great antiquity of the host 'peridotitic' diamonds (relative to the age of the associated kimberlites) and hence an extended period of residence in the sub-continental mantle. Here we report trace-element data from garnet inclusions in peridotitic diamonds from the Mir kimberlite pipe, Siberia, which appear to be inconsistent with this interpretation. The heterogeneous distribution of the trace elements both within and among discrete garnets from single diamonds are indicative of rapid crystal growth leading to solid-melt disequilibrium on a local scale. The conditions for survival of these heterogeneities within individual garnet crystals are tightly constrained by diffusion kinetics, which would rapidly homogenize the trace-element distributions in the sub-continental mantle. Thus, while it seems clear that peridotitic diamonds and their inclusions are derived from ancient lithospheric material, our data require that they crystallized shortly before the eruption of the kimberlite 360 Myr ago⁵.

Samples examined here are chrome pyrope garnets from three diamonds from the Mir kimberlite pipe, Siberia. All three diamonds were colourless, sharp-edged, slightly flattened octahedral crystals typical of the Yakutian diamond deposits⁶. No cracks were observed in any parts of the crystals, and no morphological modifications indicative of crack-healing/overgrowth were present. The initial weights of the crystals were 0.2, 0.24 and 0.9 carats for MR92/9, MR129/15 and AV49, respectively. Six discrete garnet grains from MR92/9, a large (200 × 200 × 200 µm) grain from MR129/15, and five grains from AV49 were liberated from host diamonds by burning them individually in a platinum bucket in air at 800 °C for several hours. Samples were optically examined occasionally during the procedure and liberated garnet grains were removed from the bucket. Cracks (depicted in Fig. 2) were probably induced by differential thermal expansion of the inclusion and host during liberation. Although no further studies were made on the host diamonds before liberation of inclusions, we assert that the integrity of the stones is beyond any doubt and strongly believe that the chemical characteristics reported here can be considered primary. Major-element compositions are characterized by low Ca (from 1.3 wt% CaO in MR129/15 to 2.6 wt% in AV49) and high Cr (from 7.9 wt% Cr₂O₃ in MR129/15 to 11.8 wt% in MR92/9), consistent with the general characteristics of diamond inclusion garnets of the peridotite suite⁷ of clinopyroxene-free⁸ dunite-harzburgite paragenesis⁹. Rare-earth elements (La, Ce, Nd, Sm, Eu, Dy, Er, Yb), Ti, V, Sr, Y and Zr were determined with a Cameca IMS 3f ion microprobe at Woods Hole Oceanographic Institution using techniques described elsewhere¹⁰. Analytical uncertainties involved in the present dataset are 10–20% for rare-earth elements (REE) and 5–10% for other trace elements.

Figure 1 illustrates REE variations within and among discrete garnets. There are two salient features of the data. First, general similarities exist among grains from a single diamond host. For instance, six grains from MR92/9 share strong depletion in light

TABLE 1 Trace-element abundances (p.p.m.) of representative spots in chrome pyrope garnets from Mir diamonds

Diamond grain	MR92/9			AV49					MR129/15			
	27	29*	30	14-1	14-2	15	17-3	17-5	High-Sr†		Low-Sr	
La	0.233	0.034	0.187	0.549	0.239	0.216	0.517	0.209	1.56	0.819	0.028	0.098
Ce	1.58	0.151	0.644	2.22	1.77	1.60	2.34	1.66	3.86	2.94	0.788	0.828
Nd	5.55	0.972	2.45	2.96	2.64	2.72	2.85	2.52	2.06	1.54	1.55	1.64
Sm	2.74	1.32	2.10	0.679	0.632	0.377	0.862	0.486	0.680	0.383	0.230	0.543
Eu	0.948	0.644	0.769	0.338	0.0854	0.136	0.093	0.0703	0.555	0.216	0.064	0.127
Dy	1.98	1.40	1.74	0.280	0.181	0.232	0.268	0.195	0.309	0.116	0.247	0.272
Er	0.607	0.557	0.451	0.202	0.249	0.208	0.283	0.233	0.391	0.378	0.285	0.217
Yb	0.756	0.652	0.609	0.452	0.301	0.443	0.566	0.345	0.296	0.182	0.363	0.426
Ti	1621	1542	1358	215	205	209	217	206	150	179	140	127
V	458	463	469	428	441	410	416	411	ND	ND	ND	ND
Sr	2.1	0.4	0.5	490	2.2	2.2	3.6	2.1	535	26.5	2.3	2.5
Y	5.5	4.9	4.4	3.0	1.2	1.1	2.0	1.4	1.6	0.65	0.38	0.31
Zr	89.5	82.4	67.8	6.2	7.0	7.0	7.3	6.3	2.7	2.5	1.5	1.9

* Average of five spots in grain 29.

† Two high-Sr points correspond two highest-LREE points in Fig. 3. REE analyses were made on spots within 15 µm of those for other trace elements. ND, not determined.

REE (LREE) relative to heavy REE (HREE), whereas five from AV49 all show characteristically sinuous REE patterns. Beneath the general similarities, however, significant variations are noticeable among grains from the same host and even within individual grains. For instance, MR92/9 grains show variations in La, Ce and Nd of more than a factor of five. It is also noticeable that the variations in LREE are mirrored in the Sr contents. In MR92/9, the Sr content ranges from 0.4 p.p.m. in grain no.

29 (the lowest in LREE) to 2.2 p.p.m. in grain 27 (highest in LREE). In AV49, a high-LREE spot in grain 14 (no. 14-1) has 490 p.p.m. Sr, in contrast to 2.2–2.7 p.p.m. for other spots in the same grain. The Sm/Nd ratios vary significantly within and among garnet grains. Second, differences between individual diamonds from a single kimberlite pipe are large. Additionally, concentrations of other trace elements are systematically different; for instance, Ti in MR92/9 grains varies between 1,360 and

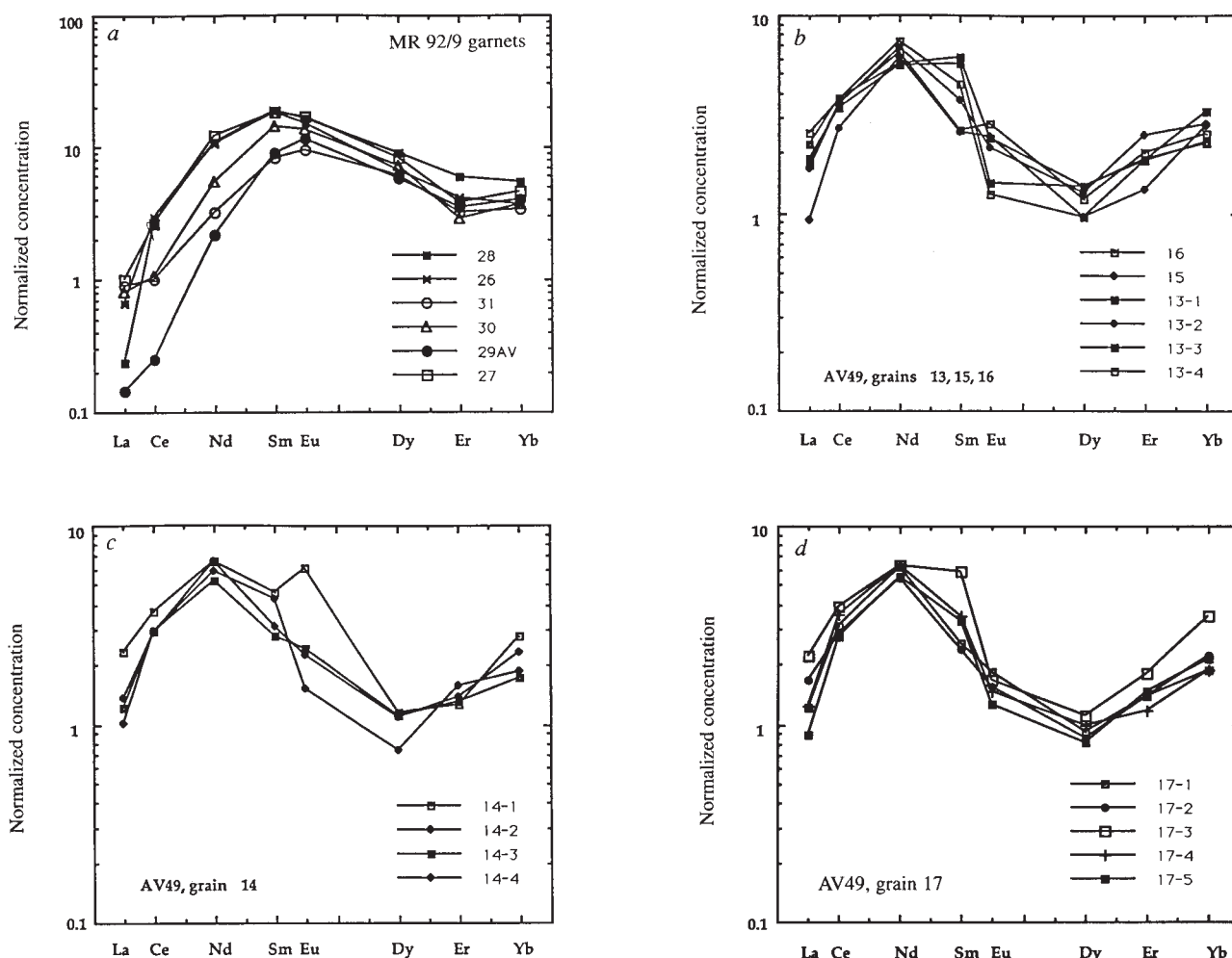


FIG. 1 Chondrite-normalized REE abundances of diamond inclusion garnets. a, Six discrete grains from diamond MR92/9; 29AV denotes

average of five spots within grain 29. b–d, Five grains from diamond AV49, showing variations within and among grains.

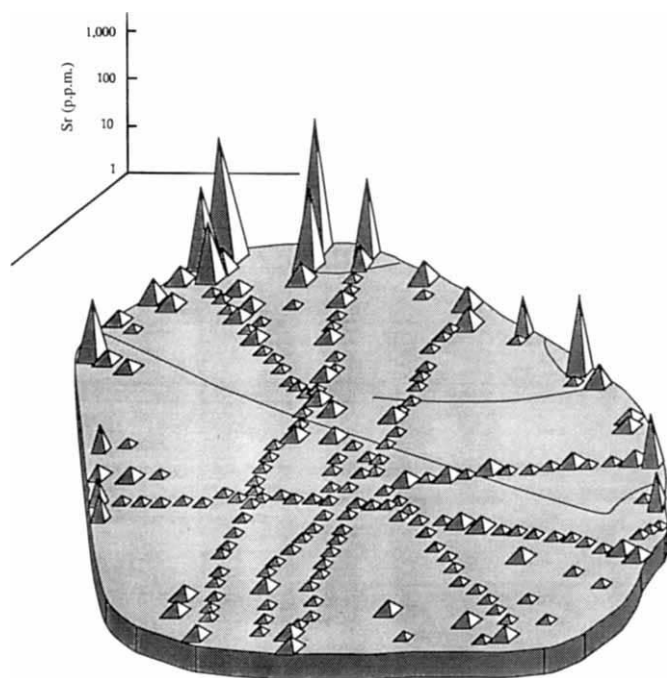


FIG. 2 The distribution of Sr across a single garnet inclusion from diamond MR129/15. Height of individual pinnacles illustrates Sr contents on a logarithmic scale. This grain is $\sim 200 \mu\text{m}$ across.

1,725 p.p.m., whereas it ranges from 196 to 220 p.p.m. in AV49 grains (see also Table 1).

Large chemical variations among discrete mineral grains from a single diamond host have been reported for garnets^{11,12} and spinels¹³ as well as Pb-isotope variations in sulphide inclusions¹⁴.

Figures 2 and 3 illustrate results of a detailed study of a single garnet inclusion from MR129/15. Figure 2 shows the distribution of Sr; it appears evident that most parts of the crystal have uniform Sr contents around 2.4 p.p.m., except for one corner of the grain where unusually high Sr contents (up to 535 p.p.m.) were found, and in steep gradients over distances of $\sim 50 \mu\text{m}$ Sr drops to the "base level" of 2.4 p.p.m. The unusual Sr concentration is not due to minute inclusion(s) of carbonate or carbonate/silicate fluid as observed in some diamonds¹⁵, because neither a decrease in Si secondary-ion intensity nor an increase in Ca intensity was accompanied by high Sr. The Ti intensity would have increased significantly had diamond inclusion fluids with high Ti contents been present¹⁵. Such an increase was not seen. Influence of unknown high-Sr surface materials(s) does not appear likely, either, because an ion beam was placed entirely within the crystal. Figure 3 shows that high Sr abundances are

accompanied by variably LREE-enriched patterns, sharing the general characteristics with the discrete garnet inclusions described above.

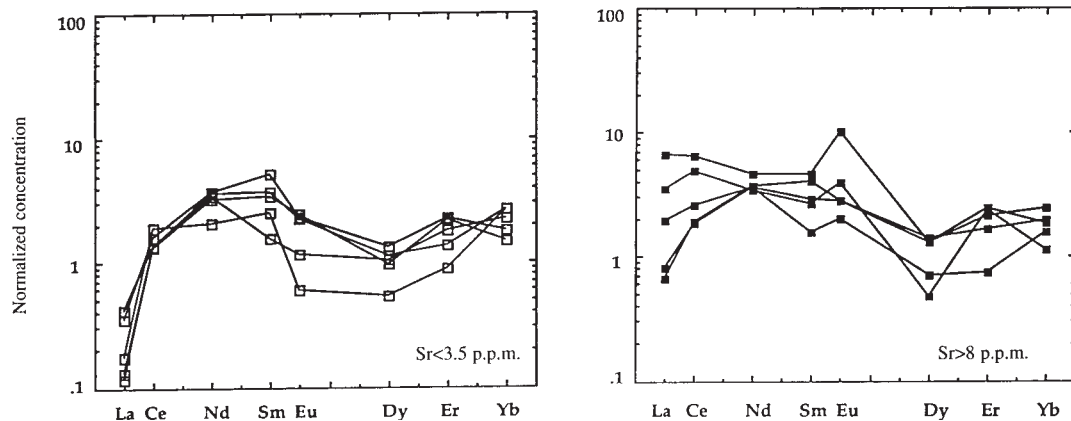
If garnets were in equilibrium with fluid/melt, the extreme Sr content (500 p.p.m.) in garnet would require a fluid/melt with an unrealistic Sr content (50 wt% Sr if a garnet-melt partition coefficient of 10^{-3} is assumed). The LREE-enriched patterns would require a fluid/melt with an extreme REE fractionation that has never been observed in nature. Alternatively, extremely high and rapidly variable Sr contents (as well as LREE-enriched patterns) could be produced by non-equilibrium distributions of these elements between garnet and fluid/melt during crystal growth. When crystal growth is sufficiently rapid relative to the rate of diffusive mass transport in the melt, incompatible trace-element concentrations in the crystal could be significantly elevated over equilibrium values. Rapid crystal growth could initiate in supercooled (supersaturated) melt, and subsequent crystal growth could be slowed down as the degree of supercooling is reduced thermally and/or chemically (constitutionally) as crystallization progresses. Initial rapid growth of garnet from LREE-enriched, high-Sr melt would thus result in unusually high Sr content (up to the Sr content of the melt) and a REE pattern that mimicks that of the melt. Continued growth would result in Sr content and changes of REE patterns more toward equilibrium values.

For MR129/15 garnet, although it is not evident where it began to grow, the distribution of Sr (Fig. 2) and the correlation between Sr and LREE (Fig. 3) suggest that the grain began rapid growth from the high-Sr corner in a non-equilibrium situation as described above. For the discrete grains from a given diamond, their temporal relationships are unknown, and it is not possible to determine how trace-element abundances varied with time during growth of the host diamonds.

The large differences in Sr contents and REE patterns between two suites of discrete garnets from a single pipe (MR92/9 and AV49; Fig. 1) may also reflect different degrees of departure from equilibrium on local scales.

The chemical heterogeneities within a single garnet inclusion reported here have important implications for the timing and mechanisms of diamond formation. Irrespective of the initial distribution of Sr, the observed gradients (Fig. 2) place a limit for a length of time in which diffusional homogenization (or relaxation) could have taken place. Experimental data¹⁶ suggest that the diffusion coefficient of Sr in garnet at temperatures appropriate for diamond formation ranges from $4.4 \times 10^{-17} \text{ cm}^2 \text{ s}^{-1}$ at $1,000^\circ\text{C}$ to $4.4 \times 10^{-15} \text{ cm}^2 \text{ s}^{-1}$ at $1,400^\circ\text{C}$. The mean temperature for diamond inclusion garnets from the Mir kimberlite, estimated from the distribution of Ni between garnet and olivine, is $1,015^\circ\text{C}$. Thus, taking $t = r^2/4D$ as the homogenization time (t) for a sphere (radius r) with diffusion coefficient D , we calculate that a grain with a radius of $100 \mu\text{m}$ would be homogenized in less than $7 \times 10^4 \text{ yr}$ at $1,000^\circ\text{C}$. There-

FIG. 3 Chondrite-normalized REE abundances across the MR129/15 garnet inclusion shown in Fig. 2. Points representing high-Sr ($\text{Sr} > 8 \text{ p.p.m.}$; left panel) and low-Sr ($< 3.5 \text{ p.p.m.}$; right panel) locations are shown to illustrate the systematic difference in REE abundances as a function of Sr.



fore the survival of the chemical heterogeneities as observed in Fig. 2 implies surprisingly low ages for garnets and thus for host diamonds of the peridotitic affinity, at least in the Siberian occurrences. This conclusion seems to be in direct conflict with the antiquity of the peridotitic diamonds based on the Sm–Nd model ages¹, and implies that garnets and host diamonds were derived from locally available materials of the ancient cratonic lithosphere^{17,18}, and were entrapped in kimberlites soon after formation in a manner discussed previously¹⁹ (a different point of view is expressed in ref. 21). The residence time of diamonds in the mantle inferred from the kinetics of nitrogen aggregation ranges from 2×10^8 to 2×10^9 years for type Ia²⁰. In view of the present results, it is evident that a multi-faceted approach, involving infrared microscopy of host diamonds and SIMS-based trace-element analysis of inclusion garnet, is necessary to obtain a definitive constraint on when these diamonds formed. □

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A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*

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GLUTATHIONE S-transferases (GSTs) are enzymes that detoxify heterocyclic compounds (xenobiotics) by covalently linking glutathione to the substrate, forming a glutathione S-conjugate^{1,2}. A glutathione pump in the vacuolar membrane of barley actively sequesters herbicide–glutathione S-conjugates; glutathionation allows recognition and entry of the conjugates into vacuoles³. The protein encoded by the *Bronze-2* gene in maize performs the last genetically defined step in anthocyanin biosynthesis, resulting in the deposition of red and purple pigments in the vacuoles of maize tissues⁴. We show here that *Bz2* encodes a GST with activity in maize, transformed *Arabidopsis thaliana* plants and *Escherichia coli*. We demonstrate that anthocyanins extracted from maize protoplasts expressing BZ2 are conjugated with glutathione, and that vanadate, a known inhibitor of the glutathione pump³ in plant vacuolar membranes, inhibits the accumulation of anthocyanins in the vacuole. These results provide a biochemical function for BZ2, and suggest a common mechanism for the ability of plants to sequester structurally similar but functionally diverse molecules in the vacuole.

The anthocyanin biosynthetic pathway is remarkably conserved among flowering plants⁵. Anthocyanins share a common biosynthetic origin and core structure (Fig. 1a), with species-specific decoration of the core by hydroxylation, methylation, sugar addition or acylation. These modifications result in the diverse red, blue and purple colours in the vacuoles of flowers, fruits and leaves. Despite the extraordinary chemical diversity of anthocyanins, these cytoplasmically synthesized molecules are all ultimately localized to the vacuole, by an unknown mechanism.

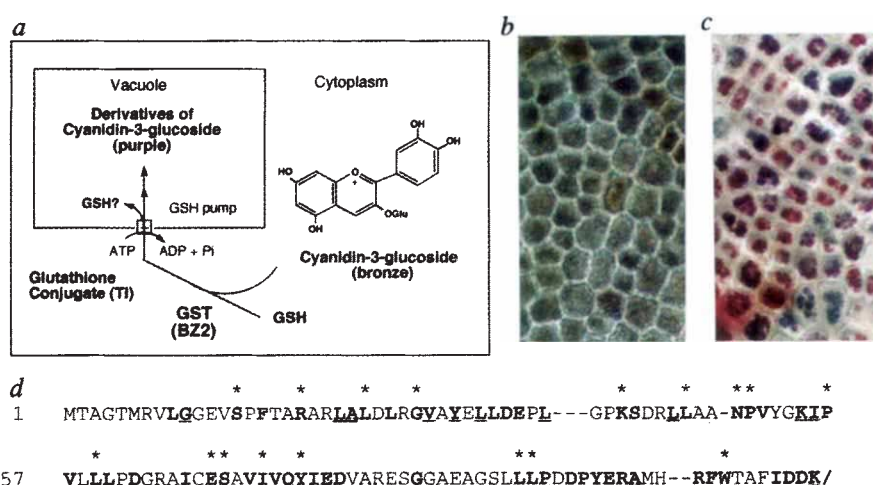
In maize, genetic and biochemical analysis has assigned specific enzymatic and regulatory roles for most genes of the anthocyanin pathway; an exception is *Bronze-2*. In *bz2* mutants, cyanidin-3-glucoside accumulates in the cytoplasm, imparting a bronze colour to tissues (Fig. 1a, b). In *Bz2* plants, anthocyanins are transferred into the vacuole (Fig. 1a, c) and become purple or red. The *Bz2* gene^{6–8} is similar to a class of plant genes induced by various stress conditions^{9–13}. Several of these share limited, but functionally significant, homology with GSTs^{14–18} (Fig. 1d). We therefore investigated whether BZ2 is a GST and how GSTs could be involved in the anthocyanin pathway.

We first compared total GST activity of isogenic maize *bz2* and *Bz2* lines. GST activity was almost twofold greater in *Bz2* plants using the conventional substrate CDNB¹⁹ (Fig. 2a). GST activity in *bz2* tissues was expected because most organisms, including maize, have multiple GST genes^{1,14–18}. We next performed transient transformation assays in maize protoplasts. We expressed *Bz2* from its native promoter (Fig. 2b, pBz2–6) by cotransformation of the maize regulatory genes *R* and *C1*, whose products together transactivate the structural genes for anthocyanin biosynthesis^{20,21}. These protoplasts accumulate anthocyanins in the vacuole within 24 hours. We also expressed *Bz2* constitutively (Fig. 2b, pCaBz2c) without activation of other anthocyanin pathway genes; these protoplasts remain colourless. In protoplasts expressing increasing amounts of BZ2, under the control of either promoter, GST activity increased up to 20-fold (Fig. 2c) within 16 hours of electroporation. Commercially available cyanidin-3-glucoside inhibited BZ2 activity against CDNB over 15-fold (not shown), indicating that cyanidin-3-glucoside is a substrate of BZ2.

We then expressed maize *Bz2* constitutively in the *Arabidopsis thaliana* *ttg* (transparent testa glabra) mutant, which lacks anthocyanin pigments²² (Fig. 2b). GST activity from control transformed, wild-type plants (WT) represents endogenous *Arabidopsis* GSTs, and is slightly higher than in control transformed *ttg* plants (Fig. 2d). Transformation of *ttg* with *Bz2* increases GST activity approximately threefold (Fig. 2d). Thus, in both maize and *Arabidopsis*, GST activity increases with BZ2 expression.

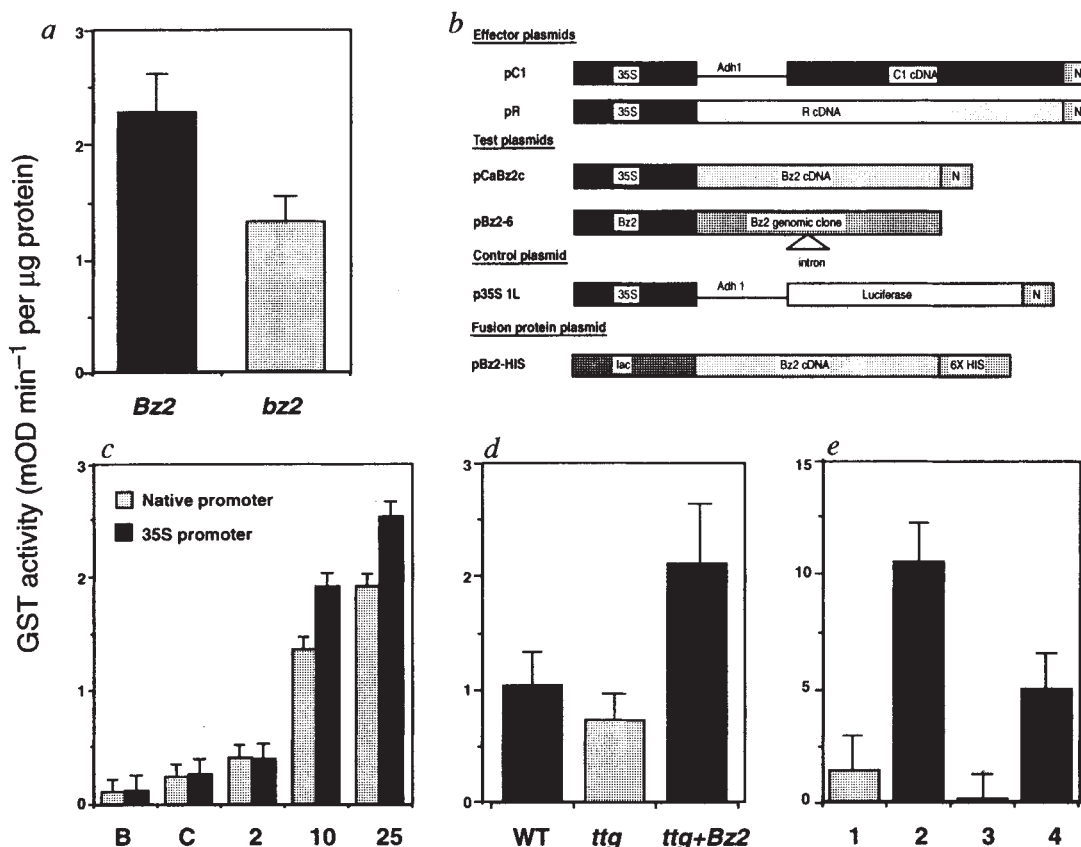
Finally, we cloned the *Bz2* cDNA into a bacterial expression vector containing a histidine affinity purification tag (pBz2-HIS; Fig. 2b). GST activity was tenfold higher in bacteria expressing BZ2 (Fig. 2e, lanes 1 and 2). After affinity purification, GST

FIG. 1 a, Anthocyanin localization to vacuoles. We propose that the GST activity of Bz2 adds a glutathione (GSH; γ -glutamylcysteinylglycine) tag to cyanidin-3-glucoside, and this glutathione conjugate is the transfer intermediate (TI) recognized by the vacuolar glutathione pump (GST pump)^{2,3}. Of the diversity of pigments in the vacuole, malonated cyanidin-3-glucoside is the most prevalent derivative in maize²⁷. The fate of the glutathione tag is unknown but, based on this report, the final purple pigment(s) retains a sulphur or cysteine of glutathione. **b**, Pigment accumulation in bz2 aleurone cells. Cyanidin-3-glucoside, a product of the sequential action of the C2, Chi, A1, A2 and Bz1 genes in maize⁴, is synthesized in the cytoplasm. **c**, Pigment accumulation in Bz2 aleurone cells. Purple, malonated cyanidin-3-glucoside is located in the vacuole of maize²⁷. **d**, Amino-acid sequence similarity between the first exon of Bz2 and other related genes: soybean *hsp26A* gene (ref. 9), tobacco GST103 and 107 (refs 10 and 11), tobacco parA (ref. 12), potato PRP1 (ref. 13), maize GST I (refs 14 and 15), III (ref. 14) and IV (ref. 16) and wheat Gst1A (ref. 17). Residues of Bz2 similar or identical to 8 of the 9 sequences are bold and indicated by an asterisk, to 7 of the 9 sequences are bold and underlined, and to at least 5 of the 9 sequences are bold only. Similarity groups used are: I, L, V, F, W, Y; K, H, R; D, E, N, Q; S, T. Dashes



indicate gaps introduced to optimize alignment. The solidus indicates the position of the exon/intron boundary of Bz2 and 4 other of the above sequences⁹⁻¹³. **METHODS.** Bz2 and bz2 aleurone cells from 4-day-old seedlings were examined by light microscopy. The BZ2 sequence was compared with reported sequences¹¹ using the BLASTp program from Intelligenetics.

FIG. 2 Bz2 is a glutathione S-transferase. a, GST activity in leaves of 8-week-old Bz2 (dark bar) and bz2 (light bar) maize plants. **b**, Constructs: pR and pC1 (refs 20 and 21), pBz2-6 (Bz2 with native promoter), pCaBz2c (Bz2 cDNA with the constitutive CaMV35S promoter), p35S-1L (luciferase control plasmid), pBz2-HIS (Bz2 cDNA with histidine affinity tag). **c**, GST activity in protoplasts electroporated with 20 μ g control plasmid (C), or with the indicated amounts (2, 10 or 25 μ g) of pBz2-6 (light bars) or pCaBz2c (dark bars). **B** refers to GST activity in boiled supernatant. **d**, GST activity in transgenic *Arabidopsis*. Wild-type (WT) and *ttg Arabidopsis* lines were transformed with a control plasmid or with pCaBz2c (*ttg* + Bz2). **e**, GST activity in *E. coli*. Cultures carrying pBz2-HIS were grown in the absence (lane 1) or presence (lane 2) of 2 mM IPTG; the BZ2 protein was also affinity purified from bacteria grown in the absence (lane 3) or presence (lane 4) of IPTG.

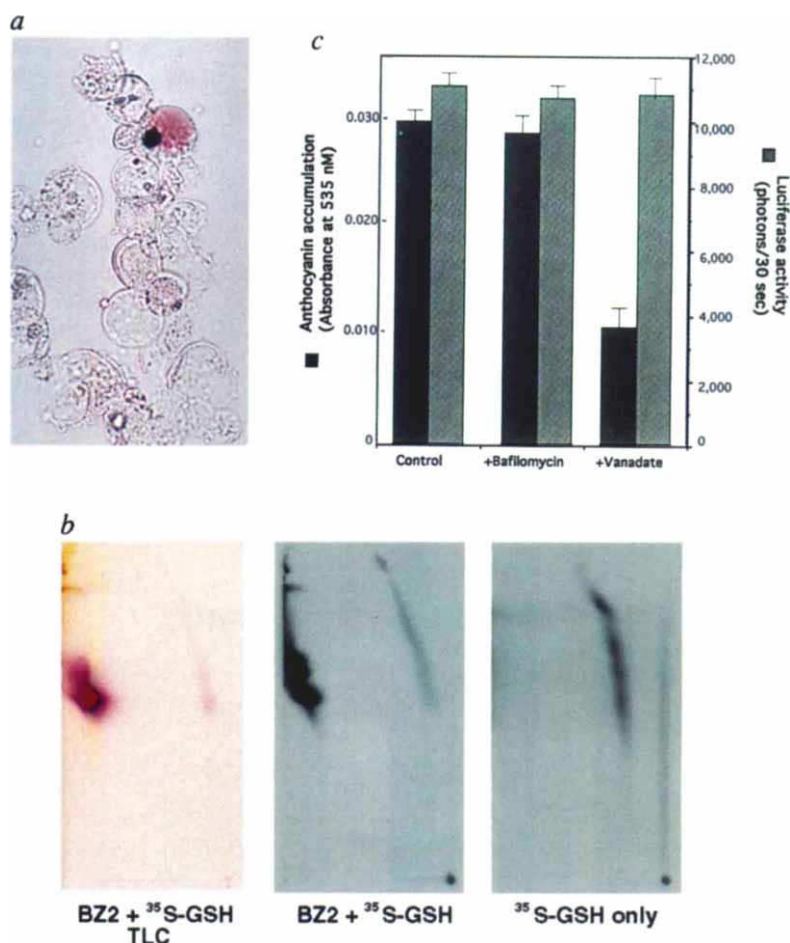


METHODS. GST activity was assayed as described¹⁹ using 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB); 100- μ l reactions were analysed on a microplate reader (Molecular Dynamics), measuring the rate of change in absorbance at 340 nm ($\Delta A_{340} \times 10^{-3}$ per min or mOD min⁻¹) per μ g protein. Maize protoplasts were electroporated as described²¹; pBz2-6 was always cotransformed with 20 μ g of pR and pC1. Wild-type *Arabidopsis* were transformed with p35S-1L, and *ttg* plants were transformed with either pCaBz2c or p35S-1L in a binary *Agrobacterium tumefaciens* vector as described^{22,30}. Transformants were self-

pollinated; 3 weeks after transfer to soil, seedling leaves from 4-6 individual progeny for each genetic construction were tested for GST activity. For BZ2 expression in *E. coli*, the Bz2 cDNA was cloned into pQE60 (Qiagen, Chatsworth, California) containing a histidine affinity tag (6X-HIS). Expression and affinity purification of pBz2-HIS was per the manufacturer's recommendation (Qiagen) except affinity purification under native conditions, which required elution from the affinity resin with 0.1 M sodium phosphate, pH 6.5, 20% glycerol and 100 mM EDTA. Bars represent the average of three independent replicates with standard deviation shown.

FIG. 3 *a*, Anthocyanin accumulation in maize protoplasts expressing Bz2, *R* and *C1*. Purple pigmentation is detected in the vacuoles of most cells within 24 hours of electroporation. *b*, Two-dimensional TLC of anthocyanins synthesized in the presence of ^{35}S -glutathione. Left panel: localization of anthocyanins on the TLC plate. Middle and right panels: localization of ^{35}S -glutathione on the TLC plate from protoplasts synthesizing anthocyanins (middle) or not synthesizing anthocyanins (right). *c*, Anthocyanin accumulation (absorbance at 535 nm, dark bars) and luciferase activity (photons per 30 s, light bars) in protoplasts treated with bafilomycin, vanadate or mock-treated (control). Luciferase activity was calculated as described²¹ from a protoplast portion taken just before anthocyanin extraction. Values represent the mean of the three independently replicated experiments with standard deviation shown.

METHODS. Protoplasts were electroporated as described in Fig. 2. For TLC analysis, 10 μCi ^{35}S -glutathione (NEN) was added to electroporated protoplasts 16 hours after electroporation. Anthocyanins were extracted 24 hours later in methanol:acetic acid:water (8:1:1). Portions (10 μl) of methanol:acetic acid extracts were spotted onto TLC plates (SigmaCell 100, Sigma) and resolved in the first dimension with Forestal (acetic acid:HCl:water at a ratio of 30:1:10) and in the second dimension with 60% acetic acid. Plates were dried and exposed to X-ray film for 24 h. For photographic purposes only, 1 μl of anthocyanin pigments extracted from dark purple maize leaves was spotted onto the TLC plates described above. However, this 'tracer' pigment did not affect the results of the TLC analysis. For inhibitor studies, protoplasts electroporated as above were treated 16 hours after electroporation with 0.5 μM bafilomycin or 5 mM sodium orthovanadate (both from Sigma) for 4 h, washed and incubated in fresh media overnight.



activity was 28-fold higher from cells expressing the BZ2 fusion protein (Fig. 2e, lanes 3 and 4).

The role of BZ2 in anthocyanin biosynthesis and its GST activity suggest that anthocyanin import into vacuoles could occur as it does for herbicide-glutathione conjugates. To look for anthocyanin-glutathione conjugates, protoplasts transformed with *R*, *C1* and *Bz2* were incubated with ^{35}S -glutathione. Anthocyanins (Fig. 3a) were extracted 24 hours later. Two-dimensional thin-layer chromatography (TLC) revealed that glutathione (GSH), or its labelled sulphur, completely comigrated with the anthocyanins (Fig. 3b, TLC plate versus middle panel). However, ^{35}S -glutathione from electroporated protoplasts not synthesizing anthocyanins (no DNA, right panel; *R* alone, *C1* alone or *Bz2* alone, not shown) or ^{35}S -GSH from the supplier (not shown) did not colocalize with ^{35}S -anthocyanins. Finally, we tested whether anthocyanin pigment accumulation could be blocked in protoplasts by the vacuolar pump inhibitors vanadate or bafilomycin. Although vanadate is a nonspecific inhibitor, vanadate sensitivity is a distinguishing feature of the glutathione pump²³. Bafilomycin is specific for the vacuolar H^+ -ATPase pump²⁴. Vanadate-treated protoplasts accumulated only one-third of the pigment of either control cells or bafilomycin-treated cells; a reporter gene construct expressing luciferase was not affected by either inhibitor (Fig. 3c).

To our knowledge, these results provide the first demonstration of a biochemical function for BZ2 and suggest a model for anthocyanin transport into vacuoles that parallels the mechan-

ism by which herbicides enter vacuoles (Fig. 1a). We propose that cyanidin-3-glucoside, conjugated with glutathione by BZ2, is recognized for transport into vacuoles by the glutathione pump; this idea is strengthened by our identification of glutathione-anthocyanin conjugates and the result that an inhibitor of this pump inhibits anthocyanin accumulation.

It is possible that the fate of anthocyanin-glutathione conjugates parallels the fate of herbicide-glutathione conjugates, many of which are metabolized to cysteine conjugates^{25,26}, and acylated with malonic acid^{25,26}. In support of this idea, it has been shown²⁷ that malonated cyanidin-3-glucoside is the major maize anthocyanin (Fig. 1a). Malonated anthocyanin derivatives are common in plants, occurring in 38% of all higher plant species²⁸, but cysteine- or glutathione-containing anthocyanin derivatives have not previously been reported.

Interestingly, the final enzymatic steps in anthocyanin biosynthesis involve cytochrome P450 monooxygenases, glucosyl-transferases and glutathione transferases, which are also the classic 'xenobiotic enzymes' induced in cells in response to toxic compounds^{1,29}. Quercetin, an anthocyanin pathway intermediate, is particularly toxic to both plants and animals⁵. We suggest that 'naturally synthesized' plant metabolites such as anthocyanins are recognized, transported and metabolized similarly to other heterocyclic compounds such as herbicides and xenobiotics²⁹. It is striking that the amino-acid sequence homology of BZ2 with other GSTs is largely confined to the N-terminal half of BZ2, encoded by the first exon (Fig. 1d). It is possible

that the first exon of *Bz2* encodes the GST activity, and that the second exon provides substrate recognition. □

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Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice

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LONG-TERM potentiation (LTP) is an enhancement of synaptic strength that can be produced by pairing of presynaptic activity with postsynaptic depolarization¹. LTP in the hippocampus has been extensively studied as a cellular model of learning and memory, but the nature of the underlying synaptic modification remains elusive, partly because our knowledge of central synapses is still limited^{2,3}. One proposal^{4,5} is that the modification is postsynaptic, and that synapses expressing only NMDA (*N*-methyl-D-aspartate) receptors before potentiation are induced by LTP to express functional AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptors. Here we report that a high proportion of synapses in hippocampal area CA1 transmit with NMDA receptors but not AMPA receptors, making these synapses effectively non-functional at normal resting potentials. These silent synapses acquire AMPA-type responses following LTP induction. Our findings challenge the view^{6–10} that LTP in CA1 involves a presynaptic modification, and suggest instead a simple postsynaptic mechanism for both induction and expression of LTP.

Whole-cell voltage-clamp recordings were obtained from hippocampal slice neurons (at the CA1/subiculum border) under visual guidance. Cells were clamped at their resting potential (~ -60 mV), dialysed with Ca^{2+} chelators (10 mM EGTA or 10 mM BAPTA, to prevent Ca^{2+} -dependent plasticity), and excitatory synaptic transmission was elicited with a weak stimulus that produced failures on about 50% of trials. Epochs of 50 to 500 trials of transmission were recorded at negative (-55 to -65 mV) and positive ($+40$ to $+60$ mV) holding potentials. As shown in Fig. 1, a higher fraction of trials produced transmission failures when cells were held at negative holding potentials compared to positive potentials ($52.7 \pm 4.6\%$ versus $20.0 \pm 2.7\%$; $n = 25$, $P < 0.002$; Fig. 1e).

Although failures have traditionally been interpreted as the failure to release transmitter, some could be due to release of transmitter at synapses without sensitive postsynaptic receptors^{2,3}. The difference in failure rates at positive and negative holding potentials could be explained if there were some synapses with only NMDA receptors. Thus transmitter released at these synapses would produce a response at depolarized potentials, with apparent failures at hyperpolarized potentials, as a result of the voltage dependence of NMDA-receptor/channel function¹¹. This model predicts that blocking NMDA receptors would prevent transmission at these synapses, and the fraction of failures at hyperpolarized and depolarized potentials should be the same. We elicited synaptic transmission by minimal stimulation in conditions similar to those above with $100 \mu\text{M}$ D,L-APV (2-amino-5-phosphonopropionic acid, an NMDA-receptor antagonist) in the bath. In these conditions, the fraction of failures was the same at hyperpolarized and depolarized potentials (Fig. 2a–d).

Could the reduction in failures at positive potentials be due solely to Cs^+ efflux through postsynaptic NMDA channels enhancing presynaptic function? To test this possibility, we monitored transmission at hyperpolarized potentials with no added Mg^{2+} in the superfusate. These conditions should allow opening of synaptically activated NMDA channels¹¹ with little leakage of internal cations. There were more failures of transmission in the presence than the absence of APV, supporting the existence of synapses with only functional NMDA receptors (Fig. 2e–h).

We estimate the fraction of release at synapses with only NMDA receptors to be 61% (Fig. 2 legend, which differs from a value $< 10\%$ observed in cultured neurons¹²). This finding indicates that measures previously used to monitor changes in presynaptic function during LTP (for example, coefficient of variance, analysis of transmission failures and frequency of miniature excitatory postsynaptic currents (e.p.s.cs) at hyperpolarized potentials) are not accurate at these synapses. Such measures will change, if, during LTP, AMPA receptors are added to synapses with only NMDA receptors. Such a mechanism has been proposed⁴, and evidence based on the coefficient of variance of AMPA and NMDA currents (an indirect measure of the number of functioning synapses with these receptors) supports such a model⁵. We have tested whether such a model can account for LTP produced by pairing low-frequency presynaptic activity (0.3–2.0 Hz, which by itself produces no potentiation; $n = 5$, data not shown) with postsynaptic depolarization (-10 mV). Such a pairing protocol may activate only some of the mechanisms recruited by tetanus-induced LTP.

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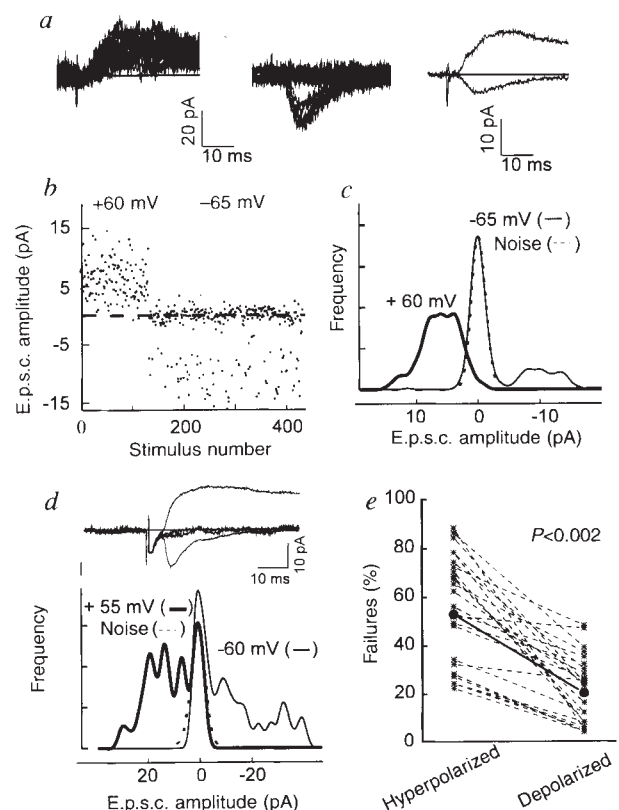
The proposed postsynaptic model for LTP predicts that many synapses which are postsynaptically silent at hyperpolarized potentials become functional with LTP. To test for this possibility we devised a protocol to stimulate just those synapses that have only NMDA receptors before LTP. We obtained whole-cell recordings holding the cell at -65 mV and stimulated an afferent pathway. The stimulus level was decreased by small increments (0.05 V) until a subminimal stimulus produced only failures for 100 consecutive trials (Fig. 3a, upper part). By depolarizing the cell to $+55$ mV it was possible to see responses (Fig. 3a, lower part). This indicates that with this protocol synapses with only NMDA responses can be activated. To test for the addition of AMPA receptors at such synapses during LTP, we conducted 39 experiments in the following manner. After gaining whole-cell access the stimulus level was gradually decreased to obtain all failures. Once failures were recorded for 100 trials, a coin toss determined if (1) the postsynaptic membrane was depolarized to -10 mV with continued presynaptic activation at the same rate and intensity for 100 trials and then returning to -65 mV, or (2) the postsynaptic membrane was kept at -65 mV and presynaptic activity was continued. Experiments were scored for the presence of responses during trials 100 to 300 for experiments with no pairing, and trials 200 to 300 for experiments with pairing (trials 100 to 200 were obscured by the pairing protocol). In 3 of 17 experiments without a pairing protocol responses were recorded after 100 failures. However, in experiments delivered a pairing protocol after 100 failures, responses were clearly visible in 12 of 22 experiments (Fig. 3; $P < 0.02$, χ^2). This indicates that functionally silent synapses can be activated by an LTP-producing pairing protocol.

If pairing-induced LTP adds AMPA receptors to postsynaptically silent synapses then there should be no change in the NMDA component of transmission after LTP. We confirmed this result^{13,14} for pairing-induced LTP which produced a $1 \pm 1\%$ increase of NMDA component with pairing ($n = 23$, $P < 0.9$, paired t -test) despite a $56 \pm 9\%$ increase of the AMPA component of transmission ($n = 23$, $P < 0.01$, paired t -test) (Fig. 4a legend). A more demanding prediction of this model is that the rate of failures should change only as measured at hyperpolarized potentials. If there is a change in presynaptic release at existing synapses, or an increase in the number of synapses releasing during LTP, then the failure rate at depolarized potentials should also change. In 12 experiments we could identify failures at hyperpolarized and depolarized potentials before and after LTP (Fig. 4b,c). As expected, there was a significant decrease in failure rate after LTP as measured at hyperpolarized potentials (before LTP, $55.8 \pm 5.8\%$; after LTP, $34.3 \pm 5.4\%$; $P < 0.002$, paired t -test). However, at depolarized potentials there was no significant change in failure rate (before LTP, $19.4 \pm 4.2\%$; after LTP, $21.5 \pm 4.7\%$; $P > 0.4$, paired t -test). This was also true in 7 experiments for which LTP was confirmed at hyperpolarizing holding potentials after the period to monitor failures at depolarizing potentials (failure rate before LTP, $23.6 \pm 4.2\%$; after LTP, $20 \pm 4.7\%$; $P > 0.4$, paired t -test). Finding no change in failure rate at depolarized potentials after LTP suggests there is no increase in presynaptic release efficacy.

Does functional recruitment of synapses by addition of AMPA receptors at pure NMDA synapses account for all pairing-induced LTP? There could be, in addition, increased AMPA responses at synapses that already had AMPA responses

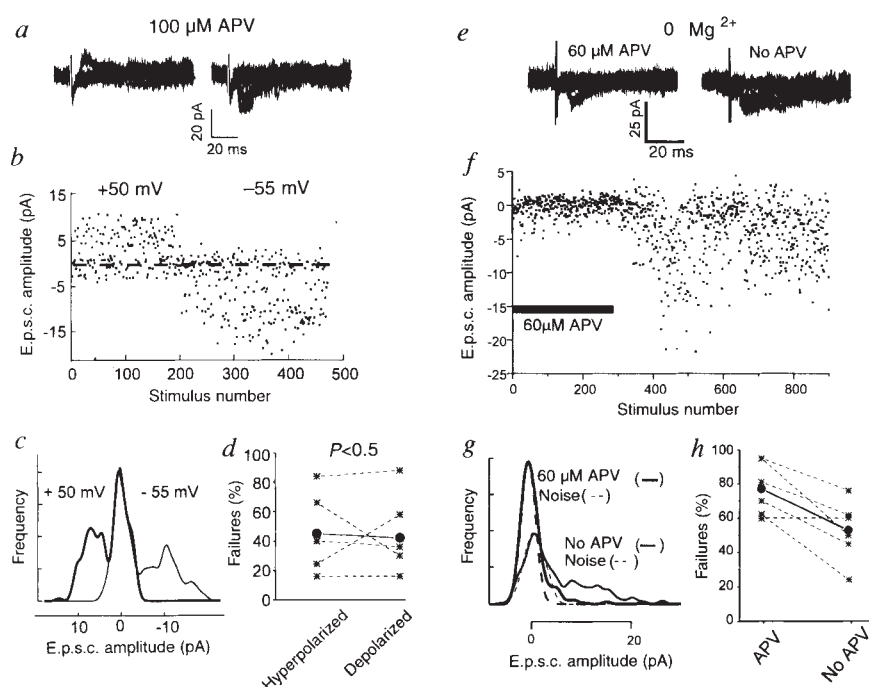
FIG. 1 Detection of transmitter release depends on postsynaptic membrane potential. **a**, A series of 15 consecutive synaptic responses recorded at a holding potential of $+60$ mV (left) and -65 mV (middle), with their averaged responses (right). **b**, Plot of excitatory postsynaptic current (e.p.s.c.) amplitude versus trial number, and **c**, e.p.s.c. amplitude distribution at $+60$ mV (thick line) and -65 mV (thin line) and noise (broken line; noise s.d. = 1.6 pA at -65 mV, n.s.d. = 2.2 pA at $+60$ mV). Note increase in frequency of events of 0 amplitude at hyperpolarized potentials. **d**, E.p.s.c. amplitude distribution density estimates of another representative experiment for epochs of e.p.s.c.s at a depolarized potential and hyperpolarized potential. Inset, averaged responses and failures at both depolarized and hyperpolarized potentials. **e**, Compiled results from 25 cells in which synaptic failures were discernible from responses. The fraction of failures recorded at hyperpolarized (left) and depolarized (right) potentials is shown (average, filled circles; individual experiments, asterisks connected with broken lines; significantly different, $P < 0.002$, paired t -test).

METHODS. Hippocampal slices were prepared from 10–18 day-old rats, the CA3 region was surgically removed, and whole-cell recordings were obtained under visual guidance from cells 2 to 3 layers below the surface. Synaptic transmission was elicited by passing current through an extracellular glass stimulating electrode placed in CA1 stratum radiatum 50 – 100 μ m from s. pyramidale. Internal solution contained 100 mM caesium gluconate, 0.6 mM EGTA (for LTP experiments), 10 mM EGTA or 10 mM BAPTA, 5 mM $MgCl_2$, 2 mM ATP, 0.3 mM GTP, 40 mM HEPES (pH 7.2 with CsOH). Bathing solution contained 119 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 26.2 mM $NaHCO_3$, 11 mM glucose, 0.1 mM picrotoxin, and gassed with $95\% O_2/5\% CO_2$ and maintained at 26 – 28 $^{\circ}C$. Complete block of responses mediated by GABA-A and GABA-B was confirmed by the lack of outward synaptic currents at a holding potential of 0 mV. Responses were amplified with Axopatch 1D, digitized (3 – 10 kHz) and stored on a computer. E.p.s.c. amplitudes were measured by placing a window of 5 – 10 ms before and around a location including the peak of the responses as recorded at hyperpolarized and depolarized potentials. For a given experiment, the same windows were used on traces recorded at all experimental conditions. The noise amplitude distribution was estimated by performing a similar measurement on traces with no stimulation. Noise s.d. calculated from such measurements was 1.0 ± 0.07 pA at hyperpolarized potentials and 2.3 ± 1.0 pA at depolarized potentials, $n = 12$. The proportion of synaptic failures for an epoch of responses was estimated by doubling the



fraction of responses with amplitude less than zero. In some experiments for which the stimulus artefact invaded the region of measurement, an average of 10 to 20 null responses (identified by eye) were digitally subtracted from all traces in the experiment. This largely removes stimulus artefact.

FIG. 2 Synapses with only NMDA receptors account for differences in failure rate under different postsynaptic manipulations. *a–d*, Transmission recorded with 100 μ M D,L-APV, an NMDA receptor antagonist, in the bath. *a*, A series of 15 consecutive responses recorded at depolarized (left) and hyperpolarized (right) potentials. *b*, Plot of e.p.s.c. amplitude versus trial number for transmission elicited at +50 mV (trials 0 to 200) and –55 mV (trials 201 to 480) in 100 μ M APV. *c*, E.p.s.c. amplitude distribution for 200 trials recorded at +50 mV (thin line) and –55 mV (thick line). Note the superposition of peaks corresponding to synaptic failures (peaks centered at 0 pA) in two conditions. *d*, Compiled results from 5 cells (as in Fig. 1*d*) indicating that the proportion of failures recorded at hyperpolarized and depolarized potentials is not significantly different ($P > 0.5$, paired *t*-test). *e–h*, Transmission recorded at hyperpolarized potentials with no added Mg^{2+} in the bath. *e*, Series of 15 consecutive traces recorded in the presence (left) or absence (right) of 60 μ M APV. *f*, Plot of e.p.s.c. amplitude versus trial number recorded in the presence (trial 0 to 300) or during wash of APV (trials 300 to 900). *g*, E.p.s.c. amplitude distribution in the presence (thick line) or absence (thin line) of APV. Amplitude distribution of noise in APV (thick broken line) largely matches responses in APV indicating high proportion of synaptic failures in APV. *h*, Compiled results from 6 cells indicating that synaptic failures were more prevalent in the presence than absence of APV ($P < 0.02$, $n = 6$, paired *t*-test). To estimate the fraction of release at synapses with only functional NMDA receptors relative to all functioning synapses, we assume that on average release probability is low²⁷ and events occur independently. Thus, $m_A = -\ln(F_H)$ and $m_A + m_N = -\ln(F_D)$, where m_A is the average release per trial at synapses with AMPA receptors, m_N is the average release at synapses with NMDA receptors and no AMPA receptors, and F_H and F_D are the fraction of responses measured as failures at hyperpolarized and



depolarized potentials, respectively. Thus $m_N/(m_A + m_N) = 1 - \ln(F_H)/\ln(F_D) = 61\%$. A similar calculation for data shown in *h* produces a value of 58%. A different interpretation of the difference in failure rate could be that there is glutamate spillover from nearby presynaptic terminals that is sufficient to activate NMDA but not AMPA receptors. This is unlikely because glutamate uptake inhibitors have little effect on NMDA-receptor responses to synaptic activation, and large effects on their responses to iontophoretic glutamate application which mimics spillover²⁸.

FIG. 3 LTP produces AMPA responses at pure NMDA synapses. *a*, Sub-minimal stimulus (see text) produces all failures at –65 mV (upper, 20 consecutive trials shown) and clear responses at +55 mV (lower, 20 consecutive trials shown). *b* and *c*, Series of 20 consecutive responses to a subminimal stimulus before (*b*, upper) and after (*b*, lower) pairing (100 trials at –10 mV). Stimulus intensity was lowered until 100 consecutive trials produced failures (*c*, trials 1 to 100). Toss of a coin determined delivery of pairing protocol (trials 110 to 205), after which responses are visible at –65 mV and persist for the duration of the experiment (trials 205 to 500). Series resistance (R_s) monitored throughout experiment (*c*, lower) indicates no change. *d*, Amplitude distribution of responses and noise before (upper) and after (lower) pairing. *e*, Collected results from 39 experiments with 100 failure trials in baseline period randomly assigned to pairing (left) or no pairing (right). METHODS. Bathing medium contained 4 mM Ca^{2+} and 4 mM Mg^{2+} . Stimuli were delivered at 1.6 Hz continuously (before placing recording pipette in the bath). After gaining whole-cell access, stimulus intensity was lowered until 100 trials produced failures. After toss of a coin (see text), pairing was delivered by depolarizing to –10 mV and continuing stimulation at the same intensity and rate (no break in stimulation). Potentiation induced with this protocol could be maintained for >30 min. Experiments were analysed off-line for presence of responses. Responses were scored if 2 or more events occurred with the same latency. In 8 of 47 experiments a single response was detected; these experiments were excluded from analysis because it was not possible to determine if responses were elicited or occurred spontaneously. The 3 experiments showing responses with no pairing had 2, 3 and 5 responses out of the 200 trials elicited after 100 failures.

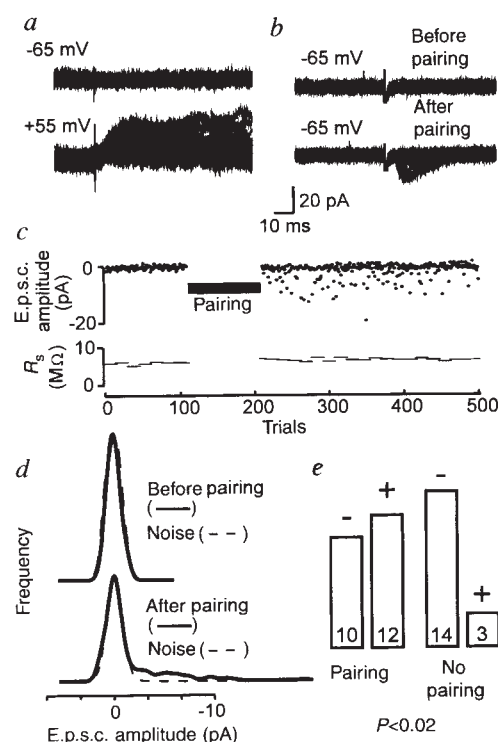
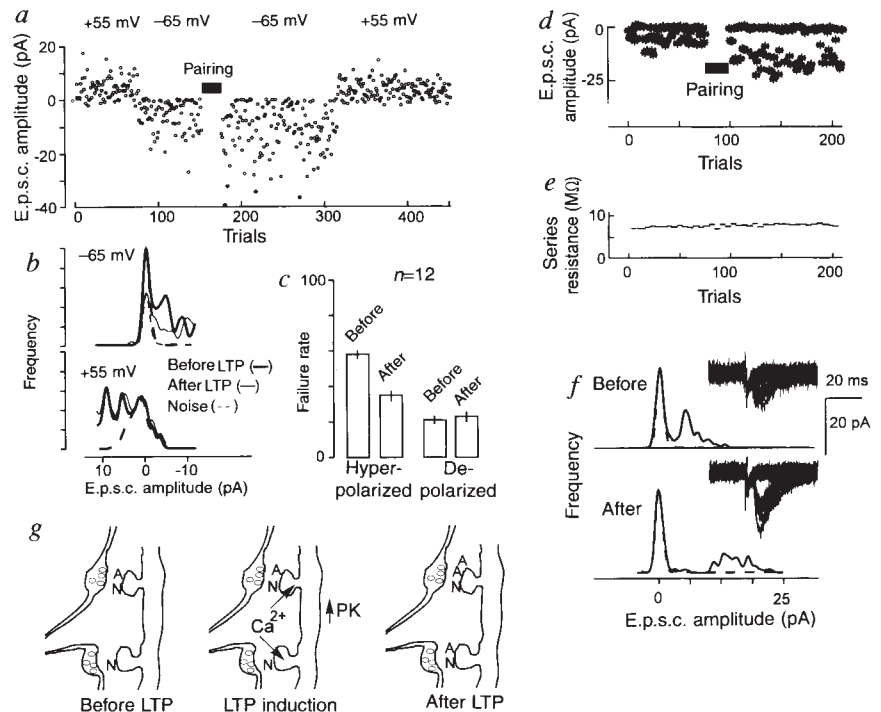


FIG. 4 Effects of LTP on failure rates indicate post-synaptic modifications. **a**, Plot of e.p.s.c. responses recorded at positive (+55 mV) and negative (-65 mV) holding potentials before and during pairing-induced LTP (-10 mV, 2 Hz stimulation). We observed no significant change in NMDA component of transmission (see text) in these or in 15 experiments for which LTP was confirmed by returning to negative potentials ($15 \pm 10\%$ increase; $n=15$, $P>0.2$, paired *t*-test). After LTP we see little enhancement of the early component at depolarized potentials. This may be because the current is dominated by NMDA currents or potentiated AMPA currents show inward rectification²⁹. **b**, E.p.s.c. amplitude density estimates before (thick line) and after LTP (thin line) at holding potential of -65 mV (upper, note change in failure peak) and +55 mV (lower, note no change in failure peak). Noise density estimate (dotted line) matches a distinct failure peak at zero amplitude. **c**, In 12 collected experiments for which failures can be identified (distinct peak at zero amplitude that matches noise amplitude), after-pairing failure rate is significantly decreased at hyperpolarized potential (see text), but is not significantly changed at depolarized potential. **d**, Plots of e.p.s.c. amplitude and **e**, series resistance versus trials before and after pairing. **f**, Amplitude distributions of e.p.s.c.s and noise before (upper) and after pairing (lower) for the same experiment. Failure rate remained the same but the mean of e.p.s.c. responses increased after pairing. **g**, Synaptic model explaining data from this study is consistent with most data in hippocampal slice LTP (see text). Left, many synapses (~60% by our estimate) have NMDA receptors with no functional AMPA receptors. Middle, during LTP, NMDA receptors open allowing entry of calcium ions and activation of postsynaptic protein kinases, leading to the functional addition of AMPA receptors to both types of synapses.



Right, after LTP induction, release of transmitter is now sensed at hyperpolarized potentials by both synapse types. Responses at synapses with AMPA responses before LTP increase by addition of more functional AMPA receptors. There could be insertion of functional AMPA receptors³⁰ or modification of existing receptors from an inactive to active mode.

before LTP. In 3 of 12 experiments there was no change in failure rate at hyperpolarized potentials despite a significant potentiation (Fig. 4d-g). In these cases LTP could not be due to addition of AMPA receptors at synapses without such receptors, or to a change in presynaptic function, because these modifications would produce a decrease in failure rate at hyperpolarized potentials. We conclude that there was an increase in AMPA responses at synapses that had AMPA responses before LTP. We expect there to be no change in failures at hyperpolarized potentials only in experiments for which there was potentiation solely at synapses with AMPA responses before LTP. Our data (Figs 1 and 2, legend) indicate that such synapses form a minority of the population. Thus, this result is expected in only a small fraction of experiments (about 1 of 3 experiments if one synapse is stimulated, fewer if multiple synapses are stimulated).

In this study, we find that a large proportion of transmission occurs at synapses with only NMDA receptors functioning. This suggests that these synapses will only transmit information while the postsynaptic cell is depolarized, perhaps by other coincident inputs acting on synapses with AMPA receptors. During development, newly formed randomly connected synapses may initially only have NMDA receptors as a means of reducing the noise that would result from activity at such randomly placed synapses. Thus, information will be transmitted at these randomly placed synapses only if presynaptic activity coincides with other, presumably functionally relevant, information.

We present several pieces of data supporting a model^{4,5} for pairing-induced LTP in which induction and expression occur postsynaptically. In this model (Fig. 4g) calcium enters synapses through NMDA receptors during LTP induction, leading to the functional addition of AMPA receptors. At synapses with only NMDA receptors before LTP (Figs 1-3a), the newly activated AMPA receptors will allow a response at hyperpolarized potentials (Fig. 3b-e) and produce a decreased failure frequency at only hyperpolarized potentials (Fig. 4a-c). At synapses with some functional AMPA receptors before LTP induction, this mechanism will produce a larger response to the release of an individual transmitter-filled vesicle (Fig. 4d-f).

This postsynaptic model is consistent with data previously interpreted to indicate presynaptic modifications during LTP, such as changes in failures, quantal content or coefficient of variance^{6,10} and frequency of spontaneous miniature responses¹⁵. In addition, this model is consistent with data supporting postsynaptic modifications during LTP, such as an increased sensitivity to exogenous transmitter¹⁶, increased AMPA binding¹⁸, a predominant change in the mean^{13,14-17} and coefficient of variance⁵ of the AMPA-receptor-mediated transmission along with no change in paired-pulse facilitation¹⁹, sensitivity to voltage-dependent calcium-channel antagonists²⁰, presynaptic calcium transients²¹, and transmitter release probability as measured with MK-801 (ref. 22; N.H. and R.M., unpublished results). Some data with tetanus-induced LTP, such as an increase in NMDA component of transmission²³⁻²⁵ and an increase in residual glutamate²⁶, or data with glutamate-induced potentiation in cultured cells, such as no change in maximal mini frequency¹⁶ suggest additional mechanisms. Thus this postsynaptic model for LTP, based on the prevalence of synapses with only NMDA receptors, can largely explain pairing-induced LTP in CA1 hippocampal slices, so there is no need for retrograde messages. □

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Properties of synaptic transmission at single hippocampal synaptic boutons

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SYNAPTIC transmission between individual presynaptic terminals and postsynaptic dendrites is a fundamental element of communication among central nervous system neurons. Yet little is known about evoked neurotransmission at the level of single presynaptic boutons^{1–5}. Here we describe key functional characteristics of individual presynaptic boutons of hippocampal neurons in culture. Excitatory postsynaptic currents (e.p.s.cs) were evoked by localized application of elevated K^+/Ca^{2+} solution to single functional boutons, visually identified by staining with the vital dye FM1-43 (refs 6, 7). Frequent repetitive stimulation produced a decline in the incidence of e.p.s.cs as the pool of releasable vesicles was exhausted; typically, recovery proceeded with a time constant of about 40 s (23 °C), and involved a vesicular pool capable of generating about 90 e.p.s.cs without recycling. At individual synapses, synaptic currents were broadly distributed in amplitude¹, but this distribution was remarkably similar at multiple synapses on a given postsynaptic neuron. The average size of synaptic currents and of responses to focal glutamate application varied fourfold across different cells, decreasing markedly with increasingly dense synaptic innervation. This raises the possibility of a very effective mechanism for coordinating synaptic strength at multiple sites throughout the dendritic tree.

In studying hippocampal synapses in culture^{1,8}, one successful case was found in which local application of hypertonic sucrose activated a single presynaptic bouton¹. We found that such activity could be isolated regularly by visualizing functional boutons with the styryl dye FM1-43 (refs 6, 7), then focally stimulating them with a puffer pipette while evoked e.p.s.cs were recorded under whole-cell voltage clamp (Fig. 1a). The puffer pipette contained 70–90 mM K^+ and 2 mM Ca^{2+} to depolarize the presynaptic membrane and allow Ca^{2+} entry, whereas spontaneous events elsewhere were minimized by superfusion with 0.2 mM Ca^{2+} external solution. The number of evoked e.p.s.cs was maxi-

mal when the puffer pipette tip was centred on the fluorescent area (Fig. 1b, trace 2), and markedly diminished if it was moved to either side (traces 1, 3). A 2 μ m offset from the optimum position was sufficient to reduce strongly the evoked response when the interbouton spacing allowed a suitable test ($n=6$). A similarly sharp fall-off was seen in postsynaptic responsivity, assessed by direct application of glutamate from an iontophoretic pipette⁹ (Fig. 1c). Small lateral movements of the pipette away from the dye-labelled bouton greatly diminished the glutamate response. Thus, with guidance from FM1-43 staining, either presynaptic terminals or postsynaptic receptor sites can be focally stimulated. The local nature of the presynaptic stimulation was corroborated by comparing fluorescent signals at adjacent single boutons (Fig. 1d). Puffer solution directed at bouton 1 decreased its fluorescence and caused the appearance of synaptic events¹⁰, whereas fluorescence of bouton 2, ~3 μ m away, remained unchanged. Out of ~80 whole-cell recordings with local K^+/Ca^{2+} stimulation, 14 satisfied this criterion for isolation of single bouton activity. When boutons were spaced by <3 μ m, the local stimulation procedure typically destined groups of 2 or 3 boutons.

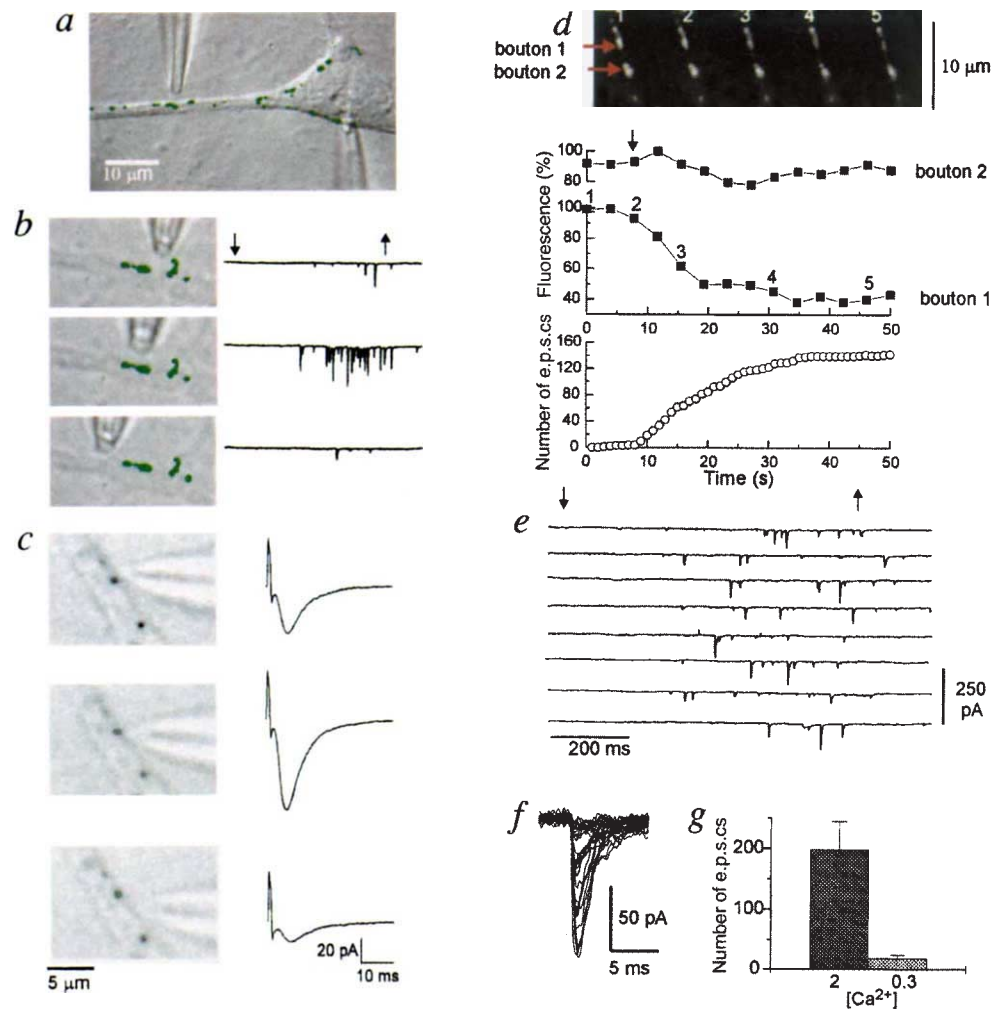
Figure 1e–g shows characteristics of the synaptic transmission from single boutons. The incidence of e.p.s.cs rose with a marked delay after initiation of pressure application and fell off sharply after its cessation (Fig. 1e), as expected for gradual settling of $[K^+]$ and $[Ca^{2+}]$ and for steep dependences of secretion on both concentrations^{11–13}. The individual e.p.s.cs are quite similar in waveform although highly variable in amplitude (Fig. 1f). The evoked events rose rapidly (20–80% rise time, 0.46 ± 0.01 ms) and decayed quickly ($\tau = 2.4 \pm 0.07$ ms, $n=186$). The incidence of evoked e.p.s.cs was highly Ca^{2+} -sensitive, as shown by alternately applying high K^+ solutions containing 2 or 0.3 mM Ca^{2+} to the same bouton (Fig. 1g). These characteristics are consistent with those expected for fast glutamatergic synaptic transmission^{14,15}.

Knowledge of the functional capabilities of nerve terminals of central nervous system (CNS) neurons is limited, although morphological characterization is extensive³. Local K^+/Ca^{2+} stimuli were effective in challenging this functional capacity¹⁶. When we presented trains of stimuli at appropriate frequencies (such as 0.2–0.5 Hz, Fig. 2a), the number of evoked events per trial declined sharply, suggesting a substantial depletion of the vesicular store. The decline is exponential at both frequencies but, at the higher frequency, the response reaches a lower steady state and requires more trials to settle. Both differences are quantitatively consistent with diminished recovery of vesicle availability during abbreviated interstimulus periods (Fig. 2 legend). The kinetics of recovery were studied by varying the rest interval t_r between trains of repetitive stimuli (Fig. 2b). The incidence of events during the second (test) train gradually recovered in degree as t_r was prolonged, but always decayed with the same time constant, consistent with a linear relationship between pool content and release rate. The recovery time course followed a single exponential, $\tau_r \sim 40$ s. These characteristics are consistent with a model (inset, Fig. 2b) which generates the smooth curves fitted to the data. For simplicity, the model divides functional synaptic vesicles into two pools, unavailable and available (including both immediately releasable and reserve vesicles^{16,17}). N designates the number of events that could be generated by the available pool at any given moment in the absence of vesicular reuse. N is governed by forward (ϕ) and reverse (ρ) transition probabilities between the pools and its initial value after a long rest is N_a . In 10 putative single bouton recordings, the average value of N_a was 93 ± 11 events (range 42–164). N_a could be interpreted as an estimate of the total number of functionally available vesicles per bouton, possibly only a lower limit because Ca^{2+} channel inactivation may have contributed somewhat to the declining response and some unitary e.p.s.cs might arise from the concerted exocytosis of multiple vesicles^{18,19}.

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FIG. 1 Stimulation and recording of synaptic activity arising from single boutons, identified, as individual puncta of FM1-43 fluorescence. **a**, Experimental configuration for focal stimulation of boutons of hippocampal neurons in culture. A fluorescence image of FM1-43 dye labelling of presynaptic boutons (green) is shown superimposed on a differential interference contrast image of a postsynaptic hippocampal neuron. A stimulating pipette points downward at the centre of an FM dye spot while a patch electrode is positioned on the cell body for whole-cell voltage-clamp recording (lower right). In most experiments, boutons close to the somatic recording site were chosen for focal stimulation to optimize the space clamp. **b**, Steep dependence of the evoked synaptic current on the position of the pipette tip relative to an area of FM1-43 staining. Vertical arrows indicate onset and termination of an 800 ms puffer application of solution containing 90 mM K^+ in place of Na^+ in normal extracellular medium (2 mM Ca^{2+}). To confine the region of stimulation, Ca^{2+} in the bulk extracellular solution was maintained at 0.2 mM. Maximum response (middle trace) obtained when the puffer pipette tip was pointed at the centre of the fluorescent area. Small lateral movements greatly decreased the efficacy of the stimulus (upper and lower traces). **c**, Inward currents at synaptic sites evoked by iontophoretic application of glutamate from a fine-tipped micropipette filled with 250 mM sodium glutamate (pH 7, ~ 100 M Ω). Iontophoretic current pulses (-100 nA, 1 ms) measured with a virtual ground amplifier. Micropipette was positioned as closely as possible to the FM1-43-stained synapse (middle) or 0.6 μ m (top) or 1.6 μ m away (bottom). The corresponding current traces include iontophoretic stimulus artefacts (upward-going pulses) and inward currents carried by non-NMDA receptor channels. It was a general finding that response amplitude fell off exponentially, decaying e-fold for lateral pipette movements of ~ 1 μ m, indicating that the glutamate-evoked response arose from stimulation of a single postsynaptic receptor cluster. **d**, Localized effect of high K^+ stimulation. Puffer pipette was directed at the centre of bouton 1. Note that the fluorescence of bouton 1 fell markedly whereas the signal at bouton 2 remained unchanged even after 50 trials of stimulation. Numbers correspond to specific time points in **d**, middle trace. Middle panel, comparison between kinetics of destaining of a single bouton and the accumulation of synaptic events. Repetitive stimulation at 0.5 Hz begins at downward arrow. Percentage fluorescence was determined for boutons 1 and 2 by integrating the fluorescent signal over appropriate pixels (same data as in **c**, with correction for fading, $\sim 1.5\%$ per exposure). The cumulative number of e.p.s.cs (bottom) generally parallels the destaining of bouton 1 (see also ref. 10). **e–g**, Physiological properties of synaptic currents originating from a single bouton. **e**, E.p.s.cs evoked from a single bouton by local application of high K^+ solution (70 mM K^+ , 2 mM Ca^{2+}) once every 10 s. Onset and termination of puffer application indicated by vertical arrows. **f**, Variable amplitude but consistently fast time course of single bouton e.p.s.cs. Same neuron as in **e**, **g**, Ca^{2+} dependence of high K^+ response, tested by alternate delivery of 70 mM K^+ solutions containing 2 mM or 0.3 mM Ca^{2+} through a theta glass pipette ($P < 0.01$ by paired *t*-test). Averages are given as mean $\pm$ s.e.m. throughout.

METHODS. The procedure for culturing hippocampal neurons was similar to that published previously⁸ with the following modification. The cells recovered by centrifugation were plated onto 12-mm round glass coverslips. Culture medium contained: minimal essential medium, glucose 0.5 g l⁻¹, glutamine 0.5 mM, NaHCO₃ 2 g l⁻¹, bovine transferrin



100 μ g ml⁻¹, insulin 25 μ g ml⁻¹, fetal calf serum (FCS) 10%. From the second day in culture, the FCS was reduced to 5% and the medium was supplemented with cytosine- β -arabino-furanoside (4 μ M) and B27 medium supplement (2%, GIBCO). The culture medium was replaced twice per week. Whole-cell recordings were obtained from spindle-shaped neurons, presumably CA1 pyramidal neurons, at room temperature (23–25 °C). The small experimental chamber (0.25 ml) was continuously perfused (0.25 ml min⁻¹) with Tyrode solution containing (in mM): NaCl 124, KCl 5, CaCl₂ 2, MgCl₂ 1, glucose 30, HEPES 25 (pH 7.4 with NaOH). Tetrodotoxin 1 μ M, D-carboxypiperazine-propyl-phosphonic acid (D-CPP) 5 μ M, and picrotoxin 50 μ M were added to block action potentials, the NMDA component of synaptic current and Cl⁻ channels mediating inhibitory synaptic currents. The patch pipette (2.2–3.5 M Ω resistance) contained (in mM): CsCl₂ 110, TEA-Cl 10, MgCl₂ 5, CaCl₂ 1, NaCl 5, EGTA 10, ATP 2, GTP 0.3, HEPES 10 (pH 7.25 with CsOH). The e.p.s.cs were blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). The hippocampal neurons were stained with 10 μ M FM1-43 in 70 mM [K⁺] for 1 min, then washed for >5 min before destaining with local stimulation. The neuron was viewed with a Zeiss IM 35 epifluorescence microscope and illuminated with a 75 W xenon lamp and neutral density transmission filter. The objective was a Nikon $\times 40$ oil immersion lens (0.8–1.3 NA). Fluorescence imaging was done with 425–480 nm excitation and 500–600 nm emission filters, with minimal exposure times (120 ms set-up time plus 33 ms per image) to reduce fading. Images were captured with a Dage 66 SIT camera and stored on a laser disc recorder. An IBM-PC 486 computer was used to control the shutter and puffer application of test solutions, and for acquisition of video frames and whole-cell current records and their off-line analysis. For studies of the time course of destaining, the boundaries of the dye-stained region were delineated for the first image, and fluorescent intensity was averaged over the pixels within the same boundary for all images. The intensity data were corrected for illumination-induced fading, using a linear time course derived from fluorescent spots well away from the site of local stimulation.

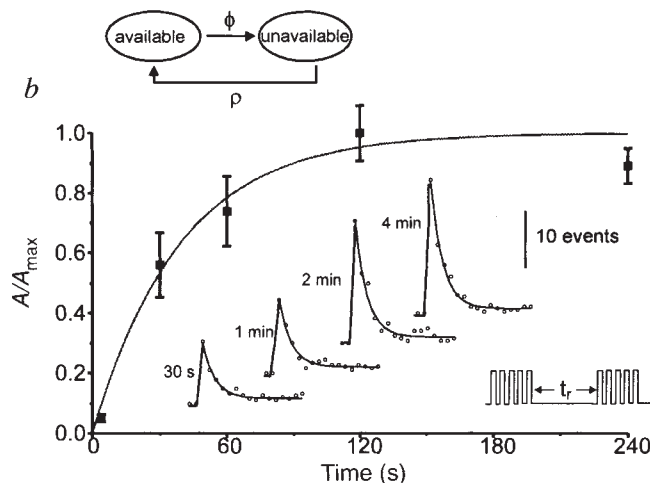
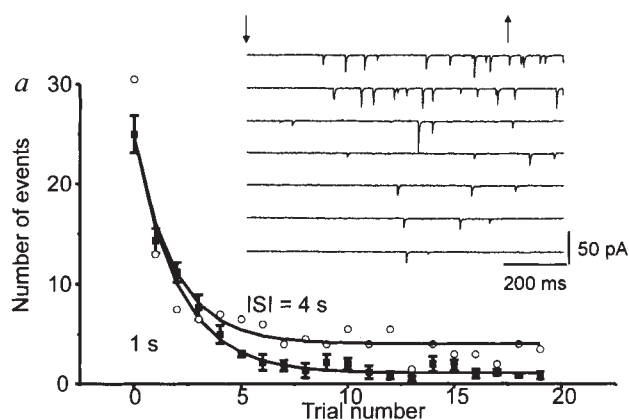
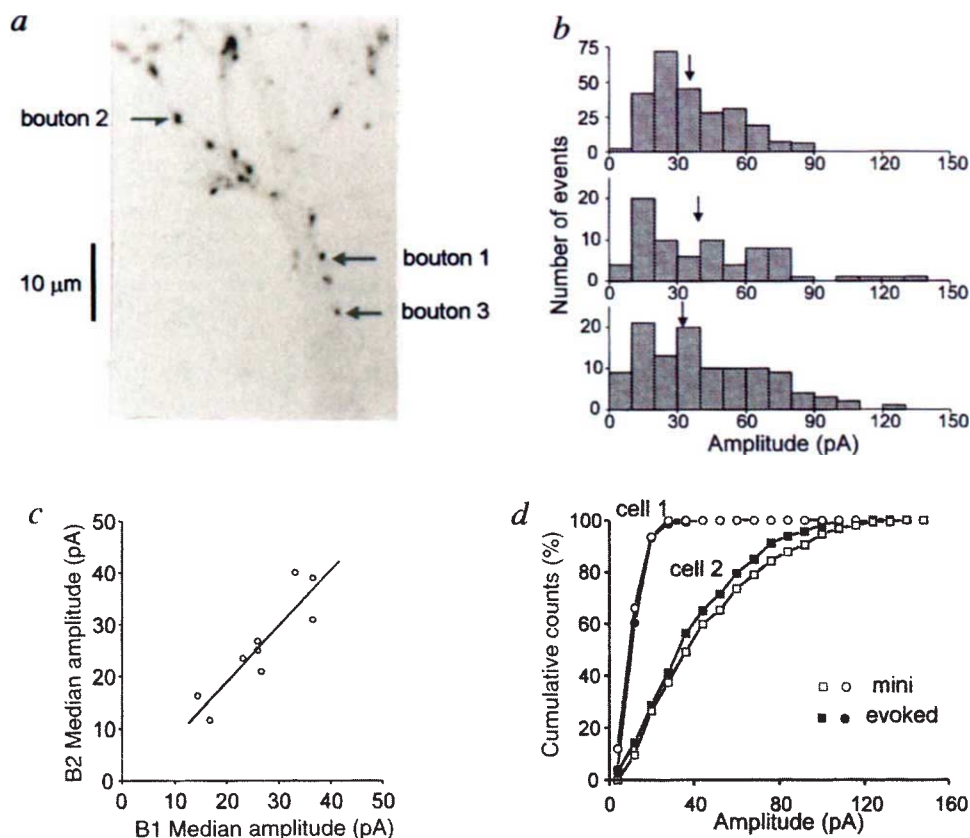


FIG. 2 Functional capabilities of hippocampal synapses delineated by single bouton stimulation. *a*, Declining response to repeated 1 s application of high K^+/Ca^{2+} solution, delivered at 0.2 Hz (interstimulus interval, ISI = 4 s) and 0.5 Hz (ISI = 1 s). Inset shows representative records for 0.5 Hz stimulation. *b*, Kinetics of recovery of responsiveness following a varying rest interval, t_r . The time course of recovery was determined by plotting the ratio of the response area after a variable rest period (A) to that after a 4 min rest period (A_{max}). Data from multiple runs from each of three individual boutons were averaged. Smooth curve is a single exponential fit ($\tau_r = 39.1$ s). Also shown are averaged responses following recovery intervals of 0.5, 1, 2 and 4 min ($n = 3$ trials at each interval). Inset, a simple model which generates the theoretical curves in *a* and *b*. Vesicles recycle between unavailable and available pools; the available pool includes both readily releasable and reserve vesicles. The forward transition (probability per trial, ϕ) incorporates vesicle docking and other steps up to and including transmitter release, whereas the reverse transition (probability per trial, ρ) represents endo-

cytosis and repriming. The variable N designates the number of events that can be generated by the available vesicular pool in the absence of endocytotic retrieval and N_0 is the initial (maximal) value of N after a long period of rest. The curves were generated by an iterative program which allows for the pulsatile nature of the stimuli; they are essentially exponentials, of the form $v = (v_0 - v_\infty)e^{-t/\tau_d} + v_\infty$, where v the number of events per trial, $v_0 = N_0\phi$, $v_\infty = N_0\phi\rho/(\phi + \rho)$, $\tau_d = (\phi + \rho)^{-1}$. The parameters for the curves are $N_0 = 66$, $\phi = 0.375$ per trial, ρ (ISI = 1 s) = 0.017 per trial, ρ (ISI = 4 s) = 4, ρ (ISI = 1 s) = 0.068 per trial (this value is plotted as the lowermost data point in *b*). In compiled results, N_0 was not correlated with the proximity of the stimulated bouton to its nearest neighbour, as expected for e.p.s.cs generated by a single bouton. An alternative to this depletion model is a scheme, discussed in ref. 16, where the response to repetitive stimuli declines because of build-up of an inhibitory factor and recovery reflects its disappearance.

FIG. 3 Comparison of single bouton responses stimulated at multiple locations on the same postsynaptic neuron. *a*, Positions of three sites of single bouton stimulation on the dendritic tree. *b*, Corresponding amplitude histograms of e.p.s.cs evoked at the three sites. Note similarity in median amplitudes (vertical arrows), 36.5, 39.1 and 31.2 pA for boutons 1, 2 and 3, respectively. $V_m = -70$ mV. *c*, Correlation between median e.p.s.c. amplitudes from multiple boutons within same cell (slope = 1.08, $r = 0.90$, $P < 0.001$). Data from pairs of boutons in 7 cells, and three boutons in 2 cells as in *a* and *b*. Note large variation in median e.p.s.c. size among different cells. *d*, Comparison of amplitude histograms (cumulative) of evoked e.p.s.cs and spontaneous miniature e.p.s.cs. Although the distributions of evoked and mini amplitudes are not significantly different for either of the two cells illustrated ($P > 0.1$ by Kolmogorov-Smirnov test), there is a striking difference between cells. In collected results from 10 cells, median amplitudes of minis were highly correlated with those of evoked e.p.s.cs (slope = 0.70, $r = 0.81$, $P < 0.005$). The spontaneous events were, on average, slightly smaller than the evoked events, and also slightly slower in both 20–80% rise time (0.59 ± 0.02 versus 0.51 ± 0.03 ms) and decay time constant (3.67 ± 0.20 versus 3.19 ± 0.19 ms) ($n = 10$ cells). These differences would be expected for spontaneous synaptic events originating from various positions within the dendritic tree, including sites where the voltage clamp is not as effective as at the site of focal stimulation.

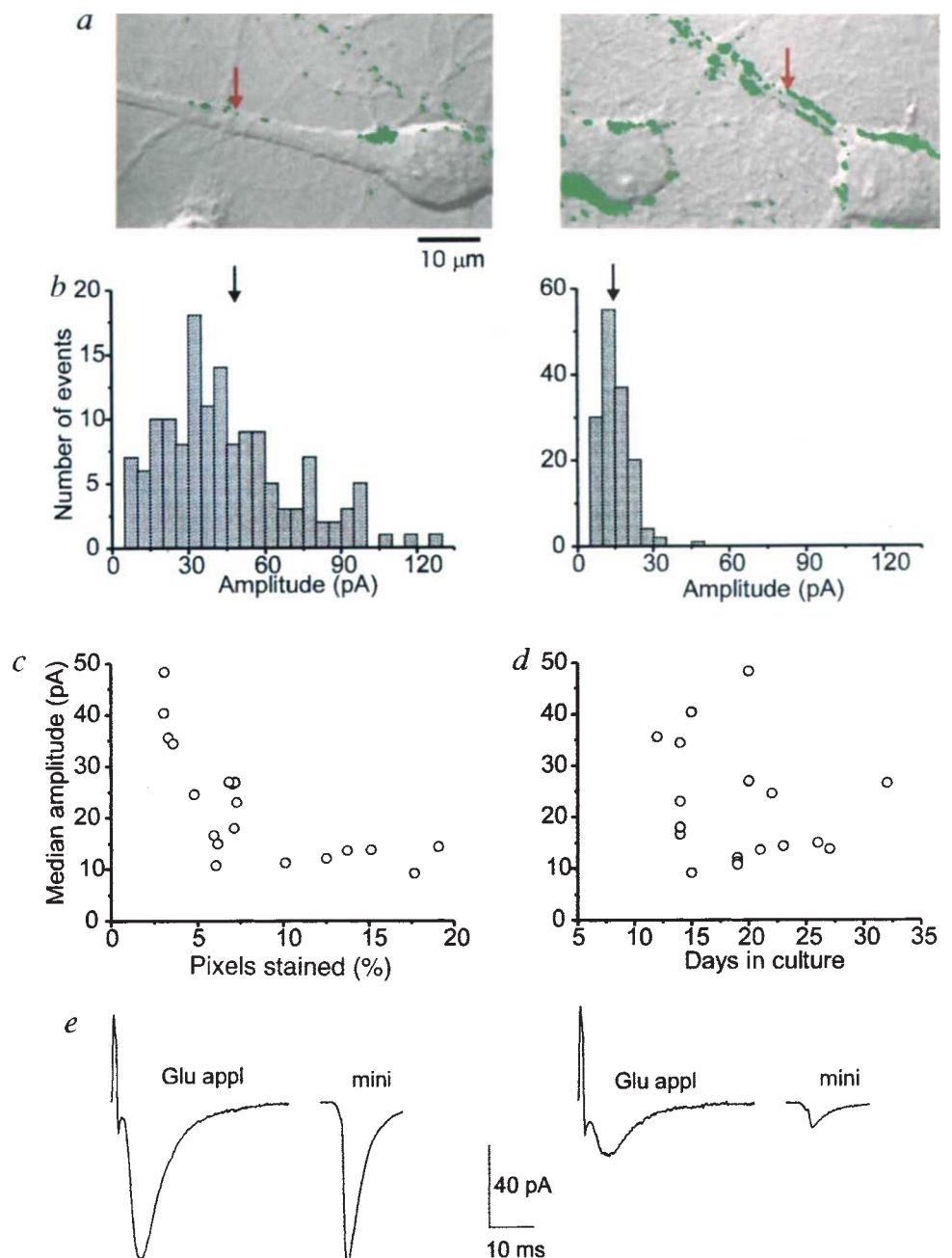


The ability to stimulate individual boutons allowed us to compare the behaviour of multiple synapses onto the same postsynaptic neuron. Figure 3 shows dye-stained boutons at three different locations on a dendrite tree (*a*) and their individual e.p.s.c. amplitude distributions (*b*). Although broad, the distributions appear similar in median amplitude (arrows). This similarity was found consistently in pairs of single bouton recordings (Fig. 3*c*): the median amplitude values for individual neurons were nearly the same even though these values varied widely from one cell to another (14–40 pA). The comparison of evoked responses has the advantage that these can be pinpointed to specific postsynaptic locations. We also compared events elicited at one synaptic site with spontaneous miniature e.p.s.cs (minis), presumably reflecting transmitter release at sites throughout the dendritic tree. The cumulative distribution of spontaneous mini size was very similar to that of K^+/Ca^{2+} -evoked e.p.s.cs (Fig. 3*d*), in cells giving small averaged synaptic responses (cell 1) or large ones (cell 2). This was a general finding (Fig. 3 legend). Evidently, the broad dispersion of mini ampli-

tudes does not reflect great variation from one synapse to the next but can be largely explained by intrinsic variability at individual synapses.

The wide range of average unitary event size across different cells prompted us to look for a possible correlation with the degree of synaptic innervation. Figure 4*a* shows representative examples of neurons with relatively low or high densities of presynaptic boutons (same cover slip). The e.p.s.cs were substantially larger in the neuron with low synapse density than in the densely innervated neuron (Fig. 4*b*). This relationship was confirmed in compiled results ($n=19$ cells, Fig. 4*c*). Median e.p.s.c. amplitude varied inversely with active synapse density as gauged by the percentage of pixels within the dendritic regions brightly stained with FM1-43. In contrast, there was no significant correlation between e.p.s.c. size and culture age ($P>0.4$, Fig. 4*d*). Although the median e.p.s.c. size was clearly larger in the more sparsely innervated cells, it was consistently small (<15 pA) when $>10\%$ of the dendrite surface area was covered with fluorescent spots (Fig. 4*c*).

FIG. 4 Single bouton responses are inversely correlated in size with the density of active synapses. *a*, Examples of cultured hippocampal neurons with low and high densities of active boutons as detected with FM1-43 staining (same coverslip). Arrows indicate the location of stimulation sites. *b*, Amplitude histograms of evoked e.p.s.cs from the neurons shown in *a*. Arrows indicate the median e.p.s.c. sizes (45 and 14 pA, respectively). *c*, Median amplitude of e.p.s.cs plotted against the density of active synapses (19 cells). Density was estimated for each cell by examining a DIC image of the recorded neuron and considering a standard length of proximal dendrite extending up to 50 μ m away from the soma. The number of pixels displaying fluorescent intensity larger than predefined threshold were expressed as a percentage of the total number of pixels in the defined dendritic area (abscissa). *d*, Median amplitude of e.p.s.cs plotted against the age of the neuronal culture. No significant correlation was found ($P=0.44$ by linear regression). *e*, Comparison of responses to focal glutamate application at a single postsynaptic locus and averaged spontaneous minis, in neurons with low synapse density (left) and high synapse density (right). Averaged traces, same iontophoretic electrode, same cover slip; see Fig. 1 for experimental details. Note that the size of the glutamate-evoked current varies greatly, in parallel with the average mini amplitude, in inverse correlation with synaptic density.



This striking pattern of behaviour could conceivably arise from either presynaptic mechanisms (variations in vesicular glutamate or the number of vesicles contributing to an e.p.s.c.), or postsynaptic ones (variations in receptor density or type). As a test for a possible presynaptic mechanism, we examined the kinetics of vesicular depletion during trains of high K^+ stimuli. No significant difference was found in N_a , ϕ or ρ in neurons with high or low innervation density (Fig. 4 legend). Thus there was no hint of a more concerted release of vesicles from a finite pool as an explanation for the larger e.p.s.c.s. To test for possible differences in postsynaptic responsiveness, we compared the responses to standardized iontophoretic application of glutamate⁹ with NMDA receptors blocked in sparsely and densely innervated cells. Figure 4e illustrates focal glutamate responses in neurons with low (3.1%, left) and high (16.2%, right) densities (same cover slip). The amplitudes of the glutamate responses were strikingly different, as with disparately sized average spontaneous minis from the same cells. In collected results, glutamate response amplitude and mini e.p.s.c. size each varied widely, but were highly correlated ($n=8$ cells, $r=0.94$, $P<0.001$). These experiments demonstrate the importance of postsynaptic factors in the variation in unitary e.p.s.c. size. The inverse relationship of postsynaptic responsiveness with synapse density may be advantageous in allowing neurons to compensate for augmented innervation, adjusting synaptic input to the dynamic range of the spike-firing decision and preventing over-excitability and toxicity. Simple competition among postsynaptic sites for a fixed number of glutamate receptors cannot be the whole explanation, because e.p.s.c. size is maintained at an apparent lower limit whereas innervation density increases twofold (Fig. 4c). The lower limit might correspond to quantal spacing in hippocampal slices^{14,20,22}.

In establishing an approach for focal stimulation of transmitter release from individual presynaptic boutons, our experiments provide new information about the functional capabilities of single synapses in responding to various patterns of stimulation. Single boutons are able to generate in the range of 40–160 e.p.s.c.s in the absence of a slow recovery process that we attribute to vesicular recycling^{6,7}. Although the distribution of unitary e.p.s.c. size is broad¹, the average size is very similar at different synaptic loci on an individual neuron. This bouton-to-bouton consistency offers functional advantages for processing of information from multiple input pathways, and augurs well for quantal analysis of evoked transmission^{14,20,22}. Future exploration of how a CNS neuron manages to control the amplitude and uniformity of the glutamate response across a multitude of postsynaptic sites will no doubt be helped by the ability to study neurotransmission at individual synapses. □

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Altered immune responses in mice lacking inducible nitric oxide synthase

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NITRIC oxide (NO) is important in many biological functions^{1–5}. It is generated from L-arginine by the enzyme NO synthase (NOS). The cytokine-inducible NOS (iNOS) is activated by several immunological stimuli, leading to the production of large quantities of NO which can be cytotoxic⁶. To define the biological role of iNOS further, we generated iNOS mutant mice. These are viable, fertile and without evident histopathological abnormalities. However, in contrast to wild-type and heterozygous mice, which are highly resistant to the protozoa parasite *Leishmania major* infection, mutant mice are uniformly susceptible. The infected mutant mice developed a significantly stronger Th1 type of immune response than the wild-type or heterozygous mice. The mutant mice showed reduced nonspecific inflammatory response to carrageenin, and were resistant to lipopolysaccharide-induced mortality.

The iNOS gene was disrupted in embryonic stem cells which were then used to produce germline chimaeras. F₁ mice (129 × MF1) heterozygous for the mutation were bred to obtain homozygotes for the disrupted iNOS gene. Loss of the wild-type allele was confirmed by Southern blotting (Fig. 1a). Homozygous animals were viable and fertile, and there was no evidence of histopathology within the major organs examined (data not shown). Interbreeding of (129 × MF1) homozygous mutant mice gave rise to a stock of mice that were used for the following experiments. Homozygous mice were compared with sex- and age-matched animals of wild-type MF1 mice or heterozygous breeding.

Peritoneal macrophages are the best-characterized source of iNOS in the mouse. Macrophages from wild-type or heterozygous mice, when stimulated with interferon(IFN)- γ and lipopolysaccharide (LPS) *in vitro*, expressed iNOS messenger RNA and iNOS protein, and produced substantial amounts of NO, measured as nitrite in the culture supernatant. In contrast, similarly stimulated macrophages from the mutant mice did not contain the normal 4.0-kilobase (kb) iNOS mRNA, but did contain an altered hybridizing species of 4.5 kb (Fig. 1b). The stimulated cells showed no detectable iNOS protein by western blot (Fig. 1c), and produced only a background concentration of nitrite, not significantly different from that produced by unstimulated cells in 24 to 48 h cultures (Fig. 1d).

The wild-type mice and the heterozygous mice were highly resistant to *L. major* infection, and all animals achieved spontaneous healing after footpad infection with 10⁶ promastigotes. In contrast, the mutant mice were highly susceptible to the infection (Fig. 2) and developed visceral disease by postinfection day 70 (data not shown). There was no significant difference in lesion size between the three groups of mice until 5 weeks after infection, indicating that the innate response, possibly under the

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influence of the *nramp* gene⁷, is unlikely to be associated with the NO effector mechanism.

Spleen cells from mutant mice infected with *L. major* contained a significantly higher proportion of CD3⁺ and CD3⁺CD4⁺ T cells than those from the infected wild-type and heterozygous mice (Table 1). Spleen cells from the infected mutant mice also showed significantly higher levels of T-cell proliferation than those from the wild-type or heterozygous mice when cultured with leishmanial antigens or concanavalin A *in vitro*. They produced more IFN- γ , but less interleukin-4 (IL-4) than those from similarly infected wild-type or heterozygous mice when stimulated with soluble leishmanial antigens *in vitro* (Table 1). Anti-leishmanial antibody titres were comparable in all the three groups of mice. The concentrations of IgG2a were greater than those of IgG1.

We also investigated the capacity of these mice to develop local inflammation. The mutant mice generated significantly less

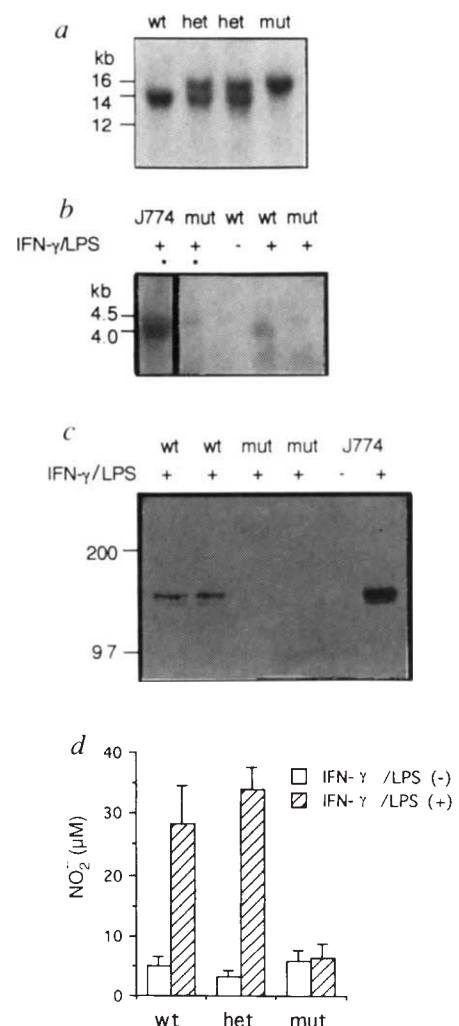
footpad swelling than wild-type mice after injection with carrageenin (Fig. 3a). Spleen cells from carrageenin-treated mutant mice also had a higher proportion of CD3⁺, CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells than those from the wild-type mice, and produced more IFN- γ than those from the wild-type mice when stimulated with concanavalin A *in vitro* (see legend to Table 1).

The mutant mice were also more resistant to LPS-induced death than wild-type mice, which developed severe loss of mass when injected intraperitoneally with LPS (Fig. 3b). The mutant mice showed an initial (first 6 to 12 h) malaise and loss of mass but recovered completely by 24 h, and showed no further sign of ill health. In contrast, wild-type mice underwent substantial loss of mass with about 50% mortality by 48 h after injection with 12.5 mg LPS per kg body mass (Fig. 3c).

Experiments using NO inhibitors have strongly implicated NO in anti-microbial and inflammatory responses⁸⁻¹⁰. However, whether iNOS is beneficial in endotoxin-induced shock has been

FIG. 1 Disruption of the murine iNOS gene by homologous recombination. A replacement type targeting construct was prepared from 129/sv genomic DNA. A single targeted clone was identified amongst 636 screened after two independent electroporations of the CGR8 embryonic stem cell line. Detailed characterization of this clone by Southern hybridization confirmed a genomic rearrangement of the iNOS gene (a), but also revealed that the expected deletion of exon 1 through to exon 5 had not occurred. Full molecular details of this event will be given elsewhere. The recombinant allele was passed through the germ line following mating of embryonic stem cell chimaeras with outbred MF1 mice (Harlan Olac, UK). Homozygous animals were then generated. The ratio of wild-type (wt): heterozygote (het): mutant (mut) mice was 25:47:21. The consequences of the rearrangement for expression of b, iNOS mRNA, c, protein, and d, production of NO were determined.

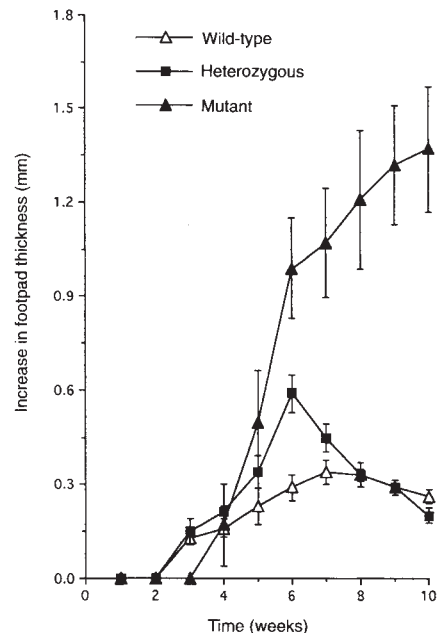
METHODS. a, DNA hybridization analysis of mouse tails from inter-cross matings of heterozygous iNOS gene mutants. DNA was restricted with *EcoRV*, fractionated by agarose electrophoresis, blotted on to nylon membrane, and hybridized with a ³²P-labelled 1.2 kbp fragment of the iNOS gene spanning exon 2 to part of the intron between exons 2 and 3. This probe hybridizes to a 14 kb fragment in both 129 and MF1 wild-type mice, which is changed to 16 kb in the mutated allele. b, RNA hybridization analysis. Peritoneal cells were collected from mutant or wild-type mice and cultured at 37 °C in medium (DMEM containing 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, 2 mM glutamine, and 10% heat-inactivated fetal calf serum) in 25-cm² tissue culture flasks (Coster) at 1 $\times$ 10⁶ cells per ml in the presence of IFN- γ (50 U ml⁻¹; gift from G. Adolf) and LPS (10 ng ml⁻¹, from *Salmonella enteritidis*, Sigma). Cells were collected 6 h (marked with asterisk) or 8 h after activation, and total RNA isolated with RNeasy (Qiagen). Samples (20 μ g each) were resolved on 1% agarose gels containing formaldehyde, transferred to a nylon filter and hybridized with iNOS complementary DNA fragment (372 bp)¹⁹ labelled with ³²P-dATP. This probe hybridizes to 4.0-kb iNOS RNA in both a murine macrophage cell line (J774, ATCC) and MF1 wild-type mice (4.5 kb in the mutated allele). This larger message appeared to be induced to a lesser extent than authentic 4.0 kb iNOS RNA. It did not translate into a biologically active iNOS protein detectable by immunoblot (c) or NO synthesis (d). c, Western blot analysis. Peritoneal cells were cultured as for b above and collected 18 h after stimulation. Cells were washed with ice-cold TBS before lysis in buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 2 mM EGTA, 10 mM NaF, 1 mM dithiothreitol, 50 μ g ml⁻¹ leupeptin, 50 μ g ml⁻¹ aprotinin, and 50 μ g ml⁻¹ PMSF. Samples (30 μ g ml⁻¹ total protein) were resolved on 7.5% SDS-PAGE gel, transferred to nitrocellulose membrane, and incubated sequentially with a monoclonal anti-iNOS antibody (MacNOS, Affinit) and then with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Scottish Antibody Production Unit, Glasgow). Protein bands were visualized with the ECL system (Amersham Life Science). A distinct band at M_r 130K was seen in the samples from wild-type mice and the control J774 cells (cultured under identical conditions), but no protein band was visible in the samples from the mutant mice. The results are representative of 4 experiments. Identical results were obtained with a polyclonal rabbit anti-iNOS antibody. d, Peritoneal cells were collected and cultured as above. Culture supernatants were collected at 24 h and 48 h after stimulation with IFN- γ and LPS, and assayed for nitrite (NO₂⁻) by the



Griess method²⁰. High concentrations of nitrite were detected in cultures of the activated cells from wild-type and heterozygous mice, whereas no nitrite (<1 μ M) was detected in the cultures of cells from the mutant mice at 24 h. By 72 h, however, a low level of nitrite was detectable in the supernatant of cells from the mutant mice. This may reflect the accumulation of nitrite produced by constitutive NOS, or the induction of constitutive NOS, which has recently been shown to be modestly upregulated by a number of reagents²¹. The data are from supernatant of 48-h cultures, representative of 4 experiments.

FIG. 2 Mice lacking iNOS failed to control *L. major* infection *in vivo*.

METHODS. Groups of 5 wild-type, heterozygous and mutant mice were infected in the footpads with 1×10^6 stationary-phase (day 7 culture) *L. major* (LV39) promastigotes. Lesion development was measured at regular intervals with a dial calliper (Schnelltaster) and expressed as the footpad thickness increase of the infected right hind foot compared with the uninfected left hind foot. The wild-type and the heterozygous mice achieved spontaneous healing, but mutant mice failed to do so. The experiment was terminated (as required by the guidelines of animal experimentation of the Home Office, UK) when the lesion in the mutant mice became ulcerated. The healing wild-type and heterozygous mice were in excellent health. When killed the mutant mice had spleens and draining lymph nodes approximately 2 to 3 $\times$ the size of those from the wild-type and heterozygous mice. The footpads of the mice we killed were removed so parasite loads could be counted. Footpad tissue from individual mice was homogenized in 10 ml PBS, and 50 μ l portions of suspension were sequentially diluted fivefold in Schneider medium (Gibco BRL) containing 20% FCS. They were placed into flat-bottom 96-well plates and incubated at 28 °C in humidified room air. Individual wells were examined daily using an inverted microscope for the presence of motile promastigotes. The geometric mean ($\log_{10}$) and standard error of the last positive reciprocal dilution for each group was calculated. The results are as follows: wild-type mice, 3.82 ± 0.8 ; heterozygous mice, 3.3 ± 0.0 ; mutant mice, 6.09 ± 0.4 .



controversial^{11,12}. Results presented here establish the crucial role of NO as an anti-microbial, an inflammatory mediator, and as a key agent in LPS-induced death. The residual but relatively low grade of inflammatory reaction and LPS-induced loss of mass observed in mutant mice in this study may be due to factors independent of NO.

An early report showed that a cloned malarial-specific Th1, but not Th2, subset of CD4⁺ T cells expressed high levels of iNOS similar to the macrophage iNOS, and that NO also inhibits the expansion of cloned Th1 but not Th2 cells¹³. Furthermore, at low concentrations NO can promote the proliferation of T cells¹⁴. The results presented here demonstrate that a low concentration of endogenously generated NO (by constitutive NOS) is probably required for Th1 cell proliferation, but a high concentration of NO produced by iNOS is necessary for the prevention of the potential overexpansion of Th1 cells, which are implicated

in a variety of autoimmune diseases. It should be noted that the preferential expansion of Th1 cells in the iNOS-deficient mice was only observed following strong antigenic challenge, and was not found in immunologically naive mice. Spleen cells from untreated mutant mice contained the same proportion of T-cell subsets as wild-type mice, and they produced concentrations of IFN- γ and IL-4 indistinguishable from those of wild-type mice following stimulation with concanavalin A *in vitro*. Thus the high concentrations of NO generated by iNOS may be important for the regulation of immune responses.

Mice deficient in iNOS provide a powerful tool that will not only facilitate formal demonstration of the effector roles of NO in microbicidal, tumoricidal, transplantation and in a range of immunopathologies, but also help to define the involvement of NO in immune regulation, immunological tolerance and antigen processing and presentation. This is highlighted by the recent

TABLE 1 T-cell analysis of spleen cells from mice infected with *L. major*

Mice	Splenic T cells (%)			Proliferation (c.p.m.)		Cytokines (pg ml ⁻¹)	
	CD3 ⁺	CD4 ⁺	CD8 ⁺	<i>L. major</i> antigen	ConA	IFN- γ	IL-4
Wild-type	16.9	7.7	4.0	576 $\pm$ 276	17,676 $\pm$ 6,010	180 $\pm$ 70	497 $\pm$ 150
Heterozygous	19.1	11.9	3.9	3,374 $\pm$ 644	9,261 $\pm$ 1,592	90 $\pm$ 30	163 $\pm$ 40
Mutant	<u>22.2</u>	<u>16.8</u>	3.1	<u>20,833 $\pm$ 5,640</u>	<u>61,812 $\pm$ 9,982</u>	<u>1,080 $\pm$ 200</u>	<u>60 $\pm$ 10</u>

Mice were killed on day 70 after infection with *Leishmania major*, as described in Fig. 2. Spleen cells were pooled from 3 mice per group and analysed for CD3⁺, CD3⁺CD4⁺ and CD3⁺CD8⁺ subsets by flow cytometry (Becton and Dickinson) using the appropriate antibodies. Figures underlined are significantly different ($P < 0.05$) from those of wild-type mice. Spleen cells were also cultured at 1×10^6 cells per ml in flat-bottom 96-well plates with graded concentrations (10^5 – 10^7 organisms equivalent per ml) of soluble *L. major* promastigote antigen (prepared by 3 $\times$ freeze-thawing and high-speed centrifugation)¹⁸, or concanavalin A (ConA) (0.5 to 5.0 μ g ml⁻¹, Sigma) for 1 to 4 days. The optimal proliferation of the cells from all three groups was on day 4 at 10^6 organisms per ml of antigen, and day 3 at 5 μ g ml⁻¹ ConA. The data are mean $\pm$ 1 s.d. ($n = 3$) of the optimal proliferation. Sera collected on postinfection day 70 were titrated against soluble *L. major* antigen-coated 96-well plate as described previously¹⁹. There was no significant difference in the anti-leishmania antibody among the three groups of mice. Titre (reciprocal dilution): total IgG, 1,000–3,000; IgG1, 10–200; IgG2a, 1,000–2,000. Spleen cells were also cultured at 1×10^6 cells per ml with 10^6 organisms equivalent per ml of soluble leishmanial antigen in 24-well plates. Supernatant was collected on days 1 to 4 and assayed¹⁹ for IFN- γ and IL-4 by ELISA using paired monoclonal antibodies (PharMingen). IFN- γ and IL-4 levels reached plateau concentrations at day 2 and day 4, respectively, and are shown here as mean $\pm$ 1 s.d. ($n = 3$). Spleen cells from mice injected with carrageenin were also analysed (21 days after injection). The mutant mice showed significantly higher levels of Th1 type of response than wild-type mice. The percentage of T-cell subsets were (mutant mice versus wild-type mice; mean $\pm$ 1 s.e.m., $n = 5$): CD3⁺, 39.32 $\pm$ 0.81% versus 19.42 $\pm$ 1.27%; CD3⁺CD4⁺, 14.69 $\pm$ 0.94% versus 8.91 $\pm$ 0.44%; CD3⁺CD8⁺, 9.38 $\pm$ 1.03% versus 2.83 $\pm$ 0.61%. Following stimulation with ConA (2.5 μ g ml⁻¹), 2×10^5 spleen cells from mutant mice produced 323 $\pm$ 9 pg ml⁻¹ IFN- γ and 61 $\pm$ 6 pg ml⁻¹ IL-4. The corresponding figures for spleen cells of wild-type mice were 69 $\pm$ 19 pg ml⁻¹ for IFN- γ and 53 $\pm$ 12 pg ml⁻¹ for IL-4. There was no significant difference in the percentage of CD3⁺, CD3⁺CD4⁺, or CD3⁺CD8⁺ subsets of T cells in the splenic population of untreated mutant mice and wild-type mice; nor was there any difference in the levels of IFN- γ and IL-4 produced by the two populations of cells following stimulation with ConA *in vitro* (data not shown).

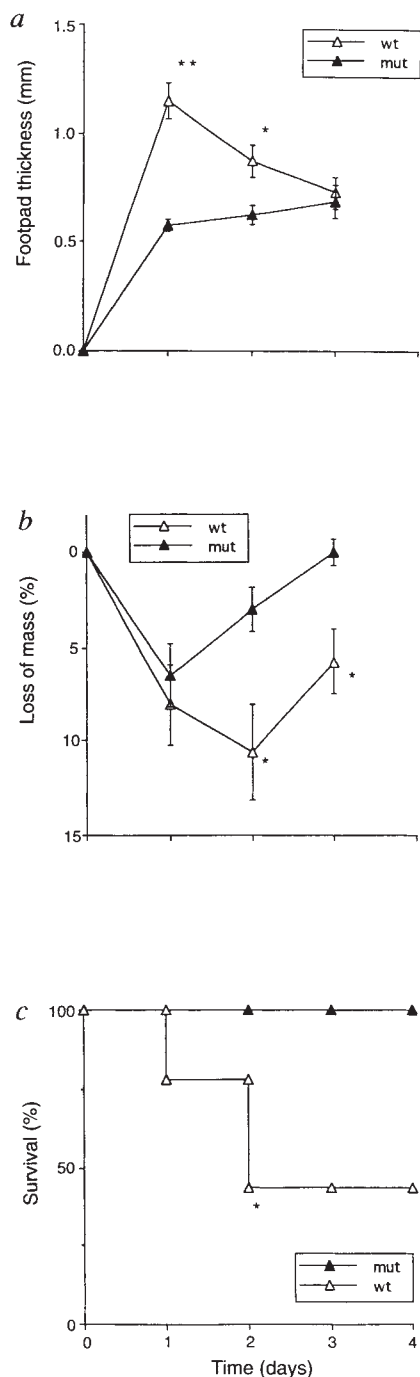


FIG. 3 Mice lacking iNOS *a*, developed significantly less inflammatory response to carrageenin, and *b*, were markedly more resistant to LPS-induced loss of mass and mortality than wild-type mice.

METHODS. *a*, Groups of 5 mice were injected in the right hind footpad with 300 μ g lambda carrageenin (Sigma) in 50 μ l PBS. The control left hind footpad was injected with 50 μ l PBS alone. Footpad swelling was measured with a dial calliper, and the data shown represent mean footpad thickness increase (right footpad—left footpad) $\pm$ s.e.m. * P = 0.02; ** P < 0.001. *b*, Groups of 5 mice were injected intraperitoneally with 10 mg per kg LPS in 0.2 ml PBS. The wild-type (wt) mice developed severe symptoms with up to 12% loss of mass. In contrast, mutant (mut) mice showed only minimum symptoms after transient loss of mass within the first 24 h, and they all recovered. * P < 0.05. *c*, Groups of 9 mice were injected with 12.5 mg per kg LPS; 5 of 9 wild-type (wt) mice died within 72 h with extensive subcutaneous haemorrhage. In contrast, all the mutant mice recovered from an initial transient loss of mass. Data are pooled from two independent experiments. * P = 0.016 (survival test).

demonstration that human monocyte/macrophages express iNOS^{15,16} and generate NO in quantities sufficient to kill *Leishmania* parasites¹⁷. □

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Herpes simplex virus turns off the TAP to evade host immunity

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MANY viruses have evolved mechanisms to avoid detection by the host immune system. Herpes simplex virus (HSV) expresses an immediate early protein, ICP47, which blocks presentation of viral peptides to MHC class I-restricted cells¹. The properties of the newly synthesized class I molecules in HSV-infected cells resemble those of cell lines deficient in the transporter associated with antigen processing (TAP) in that class I molecules are retained in the endoplasmic reticulum^{1,2}, and the heavy chain and β_2 -microglobulin subunits dissociate in detergent extracts but the complex can be stabilized by peptides¹. We show here that ICP47 binds to TAP and prevents peptide translocation into the endoplasmic reticulum.

The instability of MHC class I molecules in detergent lysates from ICP47-expressing cells suggested¹ that the class I molecules had not been loaded with peptide, which is necessary for the stable association of β_2 -microglobulin and heavy chain and subsequent transport of the class I complex from the endoplasmic reticulum (ER) to the cell surface^{3,4}. ICP47 is a cytosolic protein of relative molecular mass (M_r) 9,000 (9K) which lacks a recognizable signal sequence¹. Transport from the cytosol into the

ER of peptides destined for presentation by class I MHC is mediated by the TAP heterodimer^{5,6}. The localization of ICP47 suggested that it might interfere with the production of peptides, their delivery to the TAP transporter or the function of TAP itself.

We therefore investigated TAP transporter activity in cells expressing ICP47. The synthetic peptide TYNRTRALI (single-letter amino-acid notation), and a complex library of 2,304 peptides were used for these experiments. Both H/TYNRTRALI peptide and library peptides contain a tyrosine for radioiodination and the glycosylation consensus motif NXT, required for N-glycan-mediated retention of peptides translocated by TAP^{7,8}. TAP function requires hydrolysis of nucleoside triphosphates^{9,10,13}; however, in contrast to the results obtained in lymphoid cells⁷, we observed decreased recovery of glycosylated peptide with increasing addition of exogenous ATP to permeabilized fibroblasts (Fig. 1a). This decrease is due to peptide hydrolysis (data not shown). Without addition of nucleoside triphosphates, glycosylated peptide was recovered on concanavalin A (ConA)-coated beads in TAP-expressing fibroblasts, and the recovery was markedly reduced by hydrolysis of ATP with apyrase (Fig. 1a). To verify that peptide translocation was TAP dependent, we carried out assays in human TAP-deficient fibroblasts. Little radioactivity was recovered in fibroblast line derived from a TAP-deficient human patient¹⁴ (Fig. 1b). We confirmed the TAP dependence of transport measured in this assay using mouse embryo fibroblasts derived from TAP-positive and TAP-negative mice, and observed recovery of glycosylated peptide only in cells expressing a functional TAP complex (data not shown).

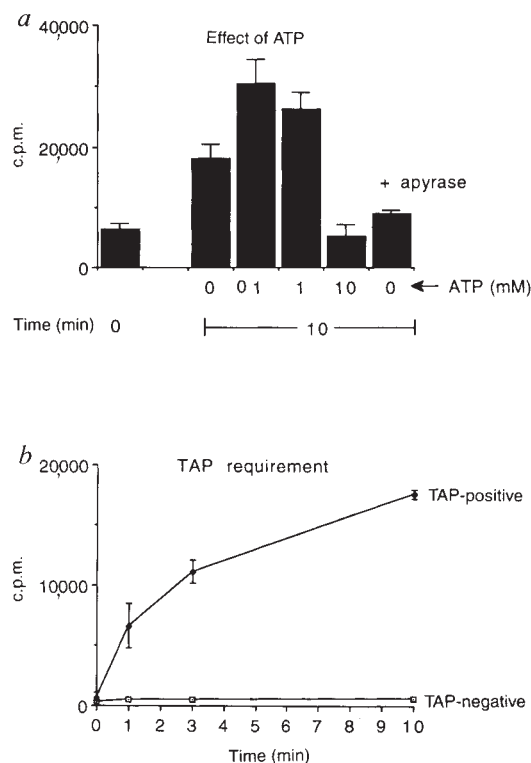
When human fibroblasts were infected with HSV type I strain F, translocation of peptide was inhibited (Fig. 2a). By contrast,

infection with HSV-1 strain R3631, which lacks the gene encoding ICP47 (ref. 15), did not affect peptide translocation. To verify the role of ICP47 in inhibition of peptide transport, we used a replication-deficient recombinant adenovirus in which the ICP47 gene, driven by the HCMV immediate early promoter, is inserted into the E1 region of the adenoviral genome, so that adenovirus proteins are not expressed¹ and ICP47 is the only viral gene product synthesized in infected cells. In cells infected with the recombinant AdICP47-1, very little of the peptide library was translocated (Fig. 2b). In contrast, in cells infected with adenovirus vectors with the E1 region deleted (AddIE1) or expressing the *lacZ* gene (Ad-*lacZ*), peptide transport was similar to that observed in uninfected cells. The translocation of peptide in AdICP47-1-infected cells was not restored by addition of either ATP or UTP (the latter can activate TAP but has less effect on cytosolic proteases than does ATP) in concentrations up to 10 mM (Fig. 2c). Translocation of the peptide TYNRTRALI results in two main species of endoglycosidase H-sensitive peptide detectable by Tris-tricine gel electrophoresis (Fig. 2d). The second species is most probably a degradation product of the input peptide, generated in the cytosol or following translocation^{16,17}. In AdICP47-1-infected cells, little peptide was recovered, but the material isolated was identical in mobility to that seen in AddIE1-infected and uninfected cells. We conclude that ICP47 inhibits TAP-dependent peptide translocation into the ER.

Does ICP47 interact directly with the TAP proteins? ICP47 was immunoprecipitated from digitonin lysates prepared from HeLa cells treated with interferon- γ (IFN- γ) and infected with AdICP47-1. Two sets of polypeptides of around 70K and 43K were coprecipitated (Fig. 3a) and comigrated with the TAP1/TAP2 complex and MHC class I heavy chain respectively (Fig.

FIG. 1 Peptide translocation in human fibroblasts is dependent on TAP and ATP. **a**, Peptide translocation requires a source of energy. ATP or apyrase at the concentrations indicated were added to human fibroblasts permeabilized with streptolysin O and incubated with the peptide library for 10 min at 37 °C. Cells were lysed and the radioactivity recovered on ConA-Sepharose was measured. The 0 min sample was washed and lysed immediately after addition of peptide at 4 °C. Results shown are the mean of duplicates. Whereas addition of exogenous ATP is inhibitory, hydrolysis of NTPs by apyrase demonstrates the dependence of the assay on a source of ATP. **b**, Peptide transport is TAP dependent. TAP-positive (CCD18-Lu) and TAP-negative (Fib-EMO) fibroblasts were permeabilized, incubated with labelled peptide library and the ConA-recovered peptide measured at various times. Peptide accumulation required an active TAP complex.

METHODS. Transport assay: Human fibroblast lines had been grown from fetal lung (HFLFB)²¹, the lung of a 2-month-old female (CCD-18Lu) (ATCC CCL 205), or from a skin biopsy of a 6-yr-old male carrying a mutation in TAP-2 rendering TAP nonfunctional (Fib-EMO)¹⁴, the gift of Henri de la Salle. They were maintained as adherent monolayers in Dulbecco's modified Eagle's medium, 5% newborn calf serum and 5% fetal calf serum (DMEM/10% serum). The cells were detached with trypsin/EDTA, washed three times in DMEM/10% serum, and washed once in transport buffer at 4 °C. (transport buffer: 130 mM KCl, 10 mM NaCl, 1 mM CaCl₂, 2 mM EGTA, 2 mM MgCl₂, 5 mM Hepes, pH 7.3 with KOH). 10⁶ cells in transport buffer were transferred to eppendorf tubes, centrifuged (3,000 r.p.m. in a microfuge), and resuspended in 80 μ l transport buffer containing 1–2 U ml⁻¹ streptolysin O (Wellcome) at 4 °C. The amount of streptolysin O used was determined for each cell line and condition of virus infection and resulted in >60% permeabilization as assessed by trypan blue exclusion. Cells were incubated at 37 °C for 10 min, then transferred to ice for addition of peptide (10 μ l), and ATP (Sigma) or apyrase (10 μ l, 50 U ml⁻¹) (Sigma) where indicated. They were then incubated for the indicated times (usually 0 and 10 min) at 37 °C. The transport assay was terminated and cells washed by addition of 1 ml 'stop buffer' (transport buffer + 10 mM EDTA), spun (14,000 r.p.m.) and lysed in 0.5% NP40, 5 mM MgCl₂, 50 mM Tris-HCl pH 7.5 (lysis buffer). After pelleting the nuclei, 100 μ l ConA-Sepharose beads (Pharmacia) were added to the supernatants and rotated gently



at 4 °C for 1 h. The beads were washed four times in lysis buffer and radioactivity measured by γ -spectrometry. A complex library of 2,304 peptides has been previously described². They were radiolabelled with ¹²⁵I by chloramine-T catalysed iodination, and used in the assay from a stock solution of 10–50 μ M in 10 mM ascorbic acid.

3a). ICP47 is small (89 amino acids) and migrates at the dyefront of these gels. TAP and class I molecules were coprecipitated from AdICP47-1-infected cells but not from AddIE1-infected cells. The identities of the 70K and 43K proteins as TAP and class I respectively were confirmed by first dissociating the immune complexes and then performing a second immunoprecipitation using anti-TAP (Fig. 3b) or anti-class I (Fig. 3c) antibodies. The presence of class I heavy chain in the complex indicates that ICP47 interacts with either the TAP complex or the TAP-class I complex^{18,19}.

TAP is expressed at relatively low levels in human fibroblasts and without IFN- γ treatment it was difficult to detect. We used recombinant vaccinia vectors expressing one (VV-TAP1 or VV-TAP2) or both (VV-TAP1&2) of the TAP subunits to assess whether the TAP heterodimer was required for the observed interaction with ICP47. Anti-ICP47 antibodies coprecipitated TAP1 and TAP2 from extracts of cells coinfecting with AdICP47-1 and VV-TAP1&2, but not when cells were coinfecting with AddIE1 and VV-TAP1&2. When cells were coinfecting with AddIE1 and VV-TAP1 or VV-TAP2, only a

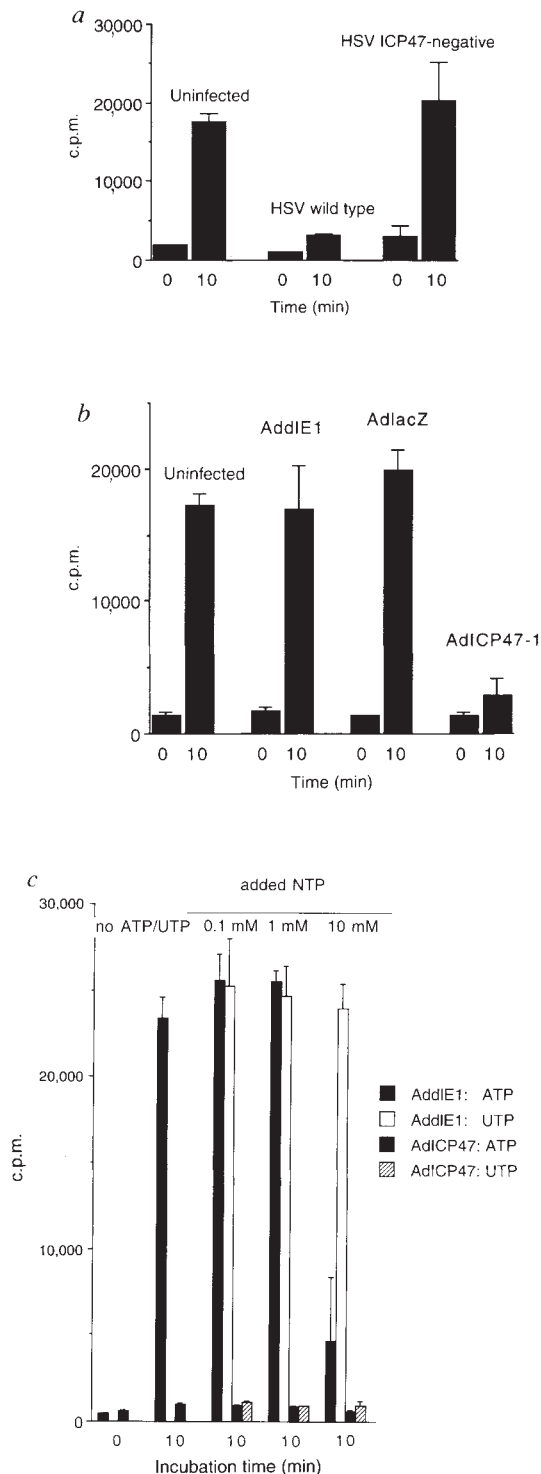
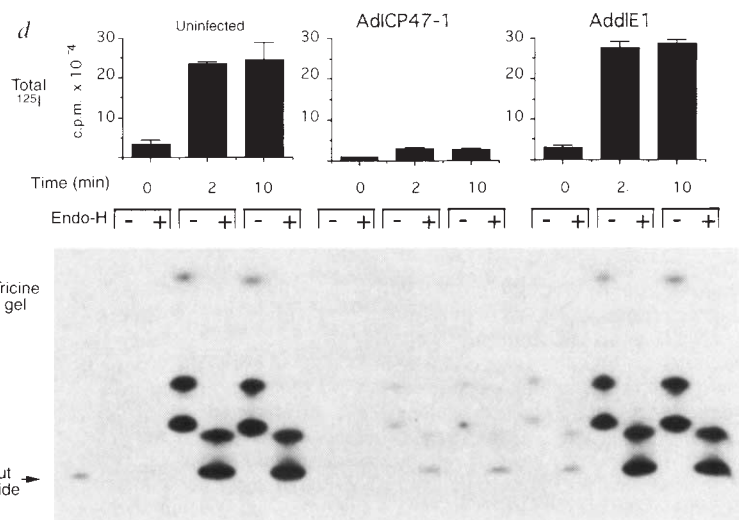


FIG. 2 ICP47 inhibits TAP-dependent peptide translocation. **a**, HSV infection inhibits peptide translocation. Human fibroblasts (CCD18-Lu) were infected using 10 plaque-forming units (PFU) per cell with wild-type HSV type I strain F or R3631, a mutant lacking the genes US11 and US12 (which encodes ICP47)¹. Nine hours later the cells were trypsinized and a transport assay with the peptide library performed as for Fig. 1. **b**, Expression of ICP47 is sufficient to inhibit TAP-dependent peptide transport. Human fibroblasts (CCD18-Lu) were left uninfected or infected using 60 PFU per cell with recombinant adenovirus expressing ICP47 (AdICP47-1), β -galactosidase (AdlacZ) or with a deletion of the E1 region (AddIE1). Sixteen hours later a transport assay with the peptide library was performed as described for Fig. 1. **c**, Neither ATP nor UTP abolishes the effect of ICP47 on transport. Transport assays were performed as in Fig. 1 using the peptide library and cells infected with the viruses indicated at 60 PFU per cell, either with no NTPs added, with ATP added, or with UTP added at the concentrations indicated. **d**, Analysis by Tris-tricine electrophoresis of peptide recovered from ConA-Sepharose. CCD-18Lu cells were infected with AddIE1, AdICP47 or left uninfected. After 18 h a transport assay with the peptide TYNRRALI was performed as described for the peptide library. After counting, peptide was eluted from the beads, half of the eluate was treated with endoglycosidase-H (endo-H) and the material analysed by Tris-tricine gel electrophoresis.



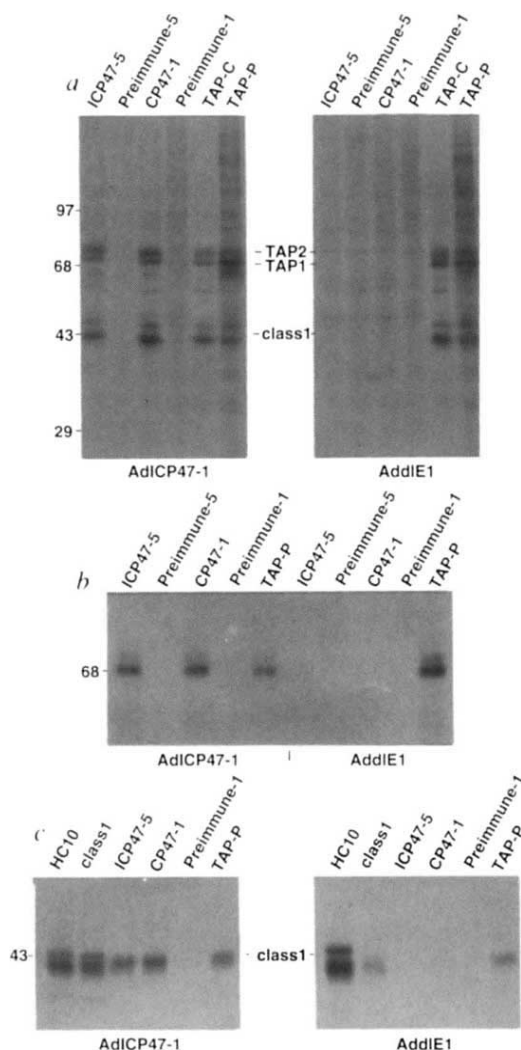


FIG. 3 ICP47 associates with the TAP complex. **a**, HeLa cells treated with IFN- γ and infected with 100 PFU per cell AdICP47-1 or AddIE1 for 36 h were metabolically labelled for 6 h and lysed in 1% digitonin. Immunoprecipitations with anti-ICP47 antisera, preimmune sera or anti-TAP sera were analysed by SDS-PAGE. **b**, **c**, Proteins immunoprecipitated as in **a** were eluted and subjected to a second round of immunoprecipitation with anti-TAP (**b**) or anti-class I (**c**) antibodies. Only in cells that expressed ICP47 was a complex of ICP47, TAP1 and class I detected.

METHODS. HeLa cells were treated with recombinant human IFN- γ (100 U per ml) for 12 h and infected with AdICP47-1 or AddIE1 using 100 PFU per cell. Thirty-six hours after infection the cells were labelled with [35 S]methionine/[35 S]cysteine (Dupont) for 6 h, and lysed in 1% digitonin/TBS (digitonin (Sigma) was dissolved at 1% w/v in 100 mM NaCl, 50 mM Tris (pH 7.5) by boiling, then cooled, filtered, and 2 mg ml $^{-1}$ BSA and 0.5 mM PMSF added). Lysates were precleared by centrifugation at 83,000g for 45 min and incubated with the following rabbit antisera: anti-ICP47-1 (raised against a carboxy-terminal peptide of ICP47), anti-ICP47-5 (raised against a full-length ICP47 produced in bacteria), preimmune sera, and anti-TAP1 antisera: anti-TAP-C 18 , anti-TAP-P 23 . Immune complexes were recovered on Protein A-Sepharose, washed four times with 0.1% digitonin/TBS; and analysed by SDS PAGE on 10% gels. Sequential immunoprecipitation: immune complexes on protein A-Sepharose were denatured by boiling for 5 min in an equal volume of 25 mM Tris pH 6.2, 2% SDS, 600 mM β -mercaptoethanol. The samples were diluted 10-fold in 1% digitonin/TBS, and immunoprecipitated with either anti-TAP-P, monoclonal antibody HC10, recognizing free HLA-B and C locus heavy chains 24 , or polyclonal rabbit serum recognizing denatured HLA class I heavy chains (class I) 25 .

small quantity of the TAP complex was precipitated by the anti-ICP47 antibodies alone, probably due to expression of endogenous TAP. Coinfection with VV-TAP1 and VV-TAP2 restored the ability of TAP to precipitate with ICP47. Thus we conclude that the interaction of ICP47 with TAP requires both TAP subunits.

We suggest that ICP47 binds to the cytosolic portion of the TAP heterodimer and thereby prevents peptide translocation. It remains a formal possibility that ICP47 interacts with MHC class I heavy chain. We consider this unlikely for two reasons. First, whereas coprecipitation of class I with TAP required the presence of only the TAP1 subunit 19,20 , coprecipitation of TAP with ICP47 required expression of both subunits. Second, upregulation of the TAPs by infection with VV-TAP1&2 dramatically increased the amount of TAP1 and TAP2 immuno-

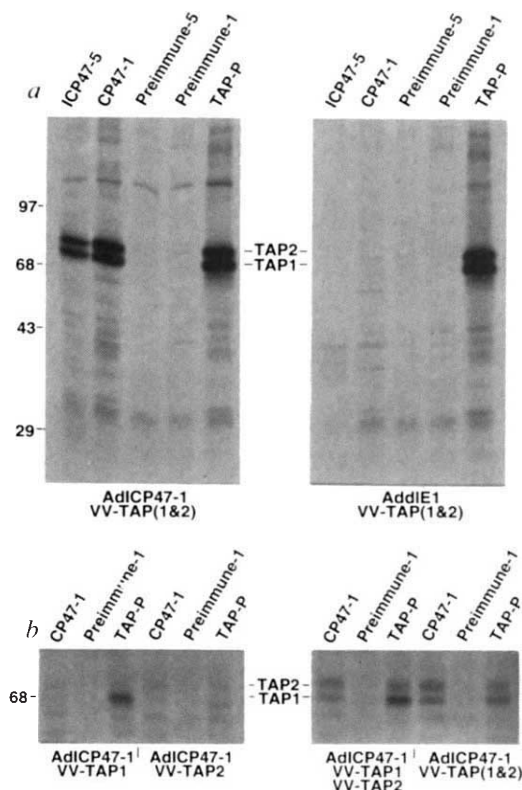


FIG. 4 Association of ICP47 and TAP requires coexpression of both TAP subunits. **a**, HeLa cells were infected with AdICP47-1 or AddIE1 using 100 PFU per cell for 36 h and then coinfecting with recombinant vaccinia viruses expressing both TAP subunits (VV-TAP1&2) for 10 h using 10 PFU per cell; then labelled with [35 S] methionine/cysteine for 4 h and immunoprecipitated as in Fig. 3 with the indicated antibodies. Class I is not detected in complexes precipitated from these cells either with anti-TAP antisera or anti-ICP47 antiserum, presumably due to the relative overabundance of the virally expressed TAP and because vaccinia shuts off host protein synthesis. **b**, HeLa cells infected with AdICP47-1 as above were coinfecting with vaccinia viruses expressing TAP1 (VV-TAP1), TAP2 (VV-TAP2), both TAP subunits (VV-TAP1&2) or simultaneously with VV-TAP1 and VV-TAP2, radiolabelled, and immunoprecipitated as above. Only when both TAP1 and TAP2 were expressed in cells did anti-ICP47 antibodies precipitate the TAP complex.

METHODS. Construction of recombinant vaccinia viruses: The single TAP subunit vectors were constructed by homologous recombination of human TAP1a or TAP2b 24 (a gift from T. Spies) cDNA in a modified form of pSC11 into the thymidine kinase region of vaccinia under the control of the p7.5 early/late promoter as previously described 27,28 . VV-TAP1&2 was constructed by inserting a plasmid containing TAP2 cDNA and the HSV thymidine kinase gene into the *Hind*III site of the VV-TAP1 and selecting for thymidine kinase expression as previously described 29 .

precipitated by anti-ICP47 antibodies, suggesting that a third molecule such as class I does not limit the association. ICP47, when overexpressed, is distributed throughout the cytosol and nucleus as detected by immunofluorescence and ICP47 produced by *in vitro* translation did not associate with ER membranes¹. These results remain to be reconciled with the present data, but explanations include the failure to detect the relatively small fraction of membrane-associated ICP47 due to the paucity of TAP subunits, and the possibility that ICP47 interacts with TAP indirectly through a cytosolic intermediary that has escaped detection.

We describe interference with peptide translocation into the ER as a novel mechanism by which viruses may evade immune control. The nature of a particular virus–host relationship is illuminated by the elements of the immune response actually targeted by the virus. For instance, four viruses are currently known to interfere post-translationally with MHC class I-restricted antigen presentation: adenovirus, murine and human cytomegaloviruses, and HSV. These viruses have some similarity in their lifestyle, in that they establish either latent or persistent infections. Herpesviruses can proceed to reactive from latency, replicate in the face of a fully primed immune system and infect new hosts. After a short period, the immune system controls the infection and little harm to the host ensues. Transmission of the virus to a new host usually occurs not from the primary infection, but from virus produced by reactivation later in life. We suggest that the ability to create this window of replicative opportunity within an immunized host is facilitated by selective avoidance of the host's CD8-positive T-cell response. □

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A viral inhibitor of peptide transporters for antigen presentation

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CYTOTOXIC T lymphocytes lyse target cells after T-cell-receptor-mediated recognition of class I major histocompatibility complex molecules presenting peptides¹. Antigenic peptides are generated in the cytoplasm by proteasomes² and translocated into the lumen of the endoplasmic reticulum (ER) by peptide transporters (TAP)^{3–6}. Herpes simplex virus (HSV) expresses a cytoplasmic protein, ICP47, which seems to interfere with such immune surveillance by mediating retention of 'empty' class I molecules in the ER^{7,8}. By expressing ICP47 in HeLa cells under an inducible promoter⁹, we show that ICP47 efficiently inhibits peptide transport across the ER membrane such that nascent class I molecules fail to acquire antigenic peptides. This inhibition was overcome by transfecting murine TAP. Further, we demonstrate that ICP47

colocalizes and physically associates with TAP within the cell. Inhibition of peptide translocation by a viral protein indicates a previously undocumented potential mechanism for viral immune evasion.

The observation that class I molecules in ICP47-expressing cells did not contain peptides⁸ suggests that ICP47 could limit the supply of peptides required for complete assembly of class I molecules in the ER, and their subsequent transport to the cell surface¹⁰. This is supported by our observation that the expression levels of class I haplotypes, at the cell surface of ICP47-expressing HeLa cells, correlated with those of respective class I haplotypes expressed in TAP-deficient cell lines (data not shown). Because ICP47 was shown to be localized in the cytoplasm⁸, it was possible that interference with antigen processing occurred before peptide translocation, e.g. at the level of proteolytic peptide generation in the cytosol. However, we observed that ICP47 prevented peptide loading of class I molecules even when proteolytic antigen degradation was bypassed by expressing minigenes encoding a class I-binding epitope (data not shown).

These observations suggest that ICP47 might interfere with peptide translocation across the ER membrane. We established a stable HeLa cell line, ICP-O20, in which ICP47 expression can be upregulated by the removal of tetracycline⁹. By using a recently developed assay for peptide transport⁵, we compared the peptide translocation activities of human TAP in ICP-O20 cells cultured in the presence of decreasing tetracycline concentrations, thus increasing the expression of the regulated gene product proportionally^{9,11}. An inverse correlation was found between levels of ICP47 expression and peptide transport activity (Fig. 1a). Even when peptide translocation activity was increased by interferon- γ (IFN- γ) which upregulates TAP expression³, ICP47 inhibited peptide translocation (Fig. 1a). Thus human TAP activity was efficiently blocked by ICP47. By contrast, when murine TAP1 and TAP2 were transiently expressed in ICP-O20 cells, high surface levels of class I

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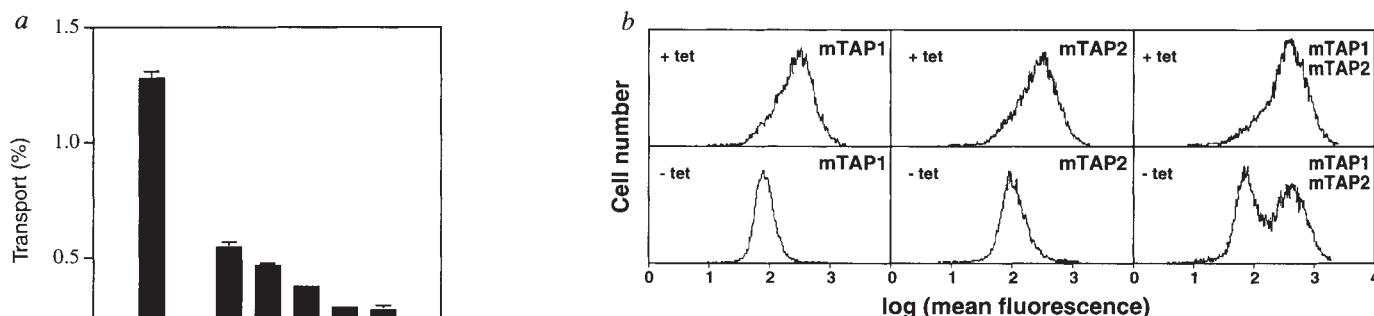


FIG. 1 *a*, Inhibition of peptide translocation by ICP47 was determined after incubating ICP-O20 cells with the indicated concentrations of tetracycline for 16 h without or with prior IFN- γ treatment. Peptide transport into the ER lumen was assayed as described previously⁵ by adding iodinated peptide RYNATRGL containing a glycosylation signal (underlined) to streptolysin O-permeabilized ICP-O20 cells and precipitating the peptides that are glycosylated in the ER with ConA Sepharose. In control experiments streptolysin O was omitted (no SLO). Results are shown as percentage of input radioactivity recovered (mean of duplicate measurements $\pm$ s.d.). *b*, FACS analysis of HLA class I surface expression in induced (-tet) and non-induced (+tet) ICP-O20 cells transfected with complementary DNA coding for murine TAP1 (mTAP1) and TAP2. Approximately 50% of cells expressed normal levels of human leukocyte antigen (HLA) class I upon cotransfection of murine TAP1 and murine TAP2. Induced ICP-O20 cells transfected with either murine TAP1 or murine TAP2 alone displayed

levels of surface-expressed class I molecules similar to those observed in induced ICP-O20 cells not transfected with murine TAP (not shown). METHODS. The gene for ICP47 was isolated from DNA extracts of HSV 1(F) by polymerase chain reaction (PCR) using specific primers, and was directly cloned into pUHG10-3 (ref. 9). HtTa cells⁹ were transfected with linearized ICP47.pUHG10-3. From several single-cell clones obtained by a selection procedure described previously¹¹, ICP-O20 was selected for further investigations. Iodinated peptides were added to 2×10^6 ICP-O20 cells permeabilized with 0.15 U streptolysin O (MUREX). After 20 min incubation at 37 °C in transport buffer⁵, cells were lysed in lysis buffer⁵ and incubated for 16 h with ConA Sepharose. ConA-bound radioactivity was measured after 5 washes in lysis buffer. For IFN- γ treatment, cells were cultured with 2,500 U ml⁻¹ of human IFN- γ (Boehringer Mannheim) for 2 days before analysis. Isolation of specific cDNAs coding for murine TAP1 and murine TAP2 (formerly ham1 and ham2) has been described^{16,17}. Both sequences were cloned into expression vector pCMU¹⁸. Plasmids were transfected as described previously¹⁸ into ICP-O20 cells either grown continuously in the presence of 1 μ g ml⁻¹ tetracycline (+tet) or after removal of tetracycline (-tet) for 16 h before transfection. FACS analysis was performed 2 days later by a standard procedure using monoclonal antibody W6/32 (ref. 19) specific for HLA class I heterodimers.

molecules were maintained (Fig. 1*b*). This reconstitution required coexpression of both murine TAP1 and TAP2 subunits. Because overexpression of human TAP by IFN- γ did not restore surface expression of class I molecules (data not shown), it is possible that ICP47 blocks the peptide translocation activities

of human TAP more efficiently than murine TAP. This is consistent with HSV-1 failing to inhibit antigen presentation in murine fibroblasts⁸.

The finding that the efficient inhibition of human TAP activity by ICP47 could be circumvented by their murine counterparts

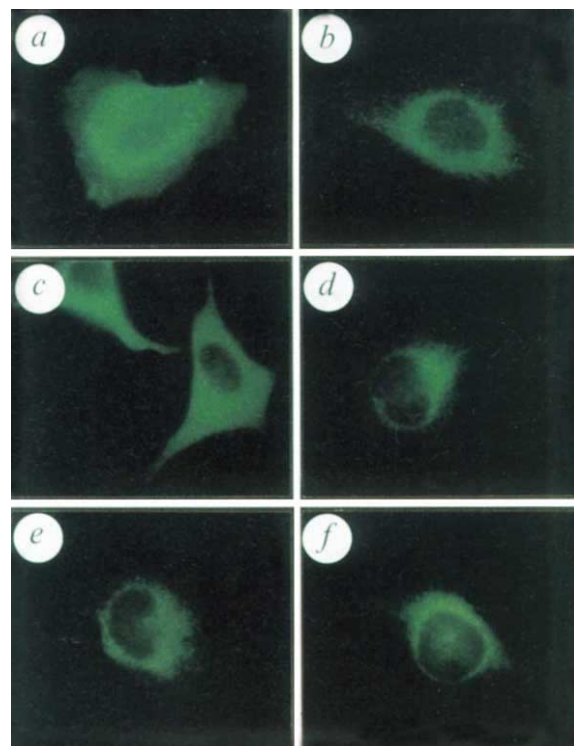
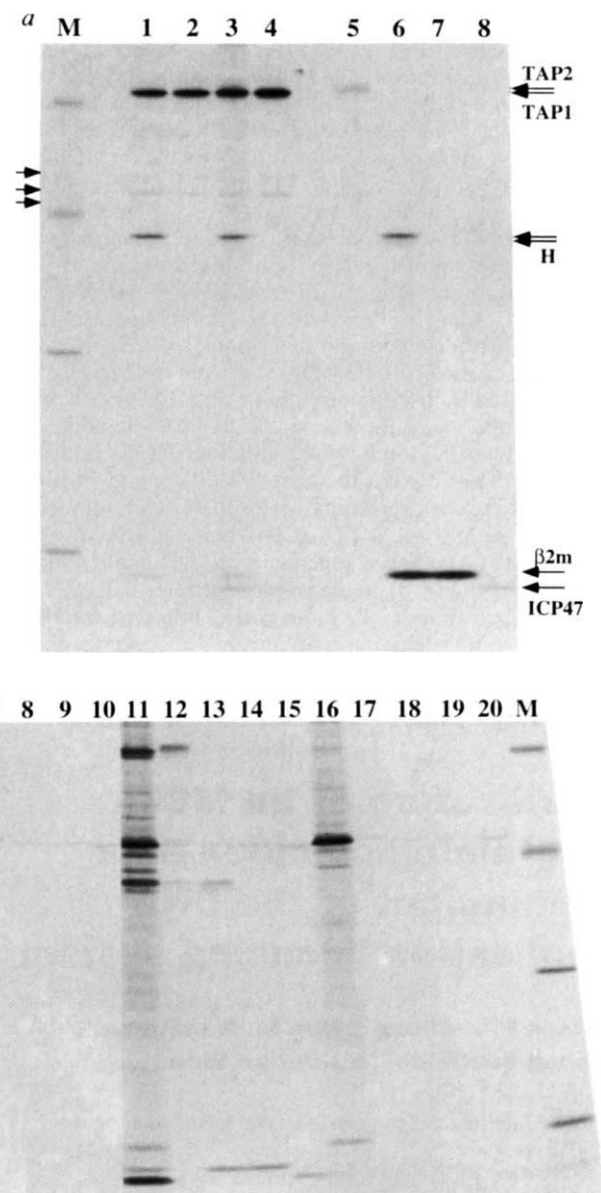


FIG. 2 Immunofluorescence analysis of transiently transfected HLA-B27 (*a*, *b*), ICP47 (*c*, *d*), ER-resident proteins (*e*)¹³, and TAP2 (*f*)²⁰. Tetracycline concentrations were 1 μ g ml⁻¹ (*a*, *e*, *f*), 0 μ g ml⁻¹ (*b*, *c*) and 0.001 μ g ml⁻¹ (*d*). Cells had been induced with IFN- γ for 2 days before analysis to increase TAP expression. HLA-B27 was selected for this analysis because it is absent from the cell surface of ICP47-expressing HtTa cells, in contrast to the residual surface expression observed for endogenous class I molecules (Fig. 1*b*). Non-transfected cells were not recognized by anti-ICP47-C or anti-HLA-B27 antibodies (not shown). Without IFN- γ treatment, anti-TAP2 showed the same immunofluorescence pattern, but with diminished intensity (not shown). METHODS. Anti-ICP47-C antiserum was raised in rabbits against synthetic peptides corresponding to the C-terminal 20 residues of ICP47. Anti-HLA-B27 monoclonal antibody was purchased from Immunotech. Immunofluorescence was performed as described previously¹⁸.

FIG. 3 a, Immunoprecipitations by a TAP1-specific monoclonal antibody²² from 1% NP40 (lanes 2 and 4) or 1% digitonin (lanes 1 and 3) lysates of non-transfected HtTa cells (lanes 1 and 2) or induced ICP-O20 cells (lanes 3 and 4). Immunoprecipitated material in lane 3 was reprecipitated by a monoclonal antibody to TAP2 (ref. 20) (lane 5), a rabbit antiserum specific for HLA class I heavy chain and β_2 -microglobulin (lane 6)²¹, a β_2 -microglobulin-specific antiserum (lane 7)²¹, or anti-ICP47-C antibody (lane 8). **b**, Anti-ICP47-C immunoprecipitations from 1% NP40 (lanes 1 and 6) or 1% digitonin (lanes 11 and 16) lysates of induced ICP-O20 cells (lanes 1 and 11) or HtTa cells (lanes 6 and 16). Reprecipitations with anti-TAP2 (lanes 2, 7, 12 and 17), anti-class I heavy chain and β_2 -microglobulin (lanes 3, 8, 13 and 18), anti- β_2 -microglobulin (lanes 4, 9, 14 and 19), or anti-ICP47-N (lanes 5, 10, 15 and 20) antibodies are shown to the right of the corresponding immunoprecipitation, as in **a**. The positions of TAP1, TAP2, class I heavy chain (H), β_2 -microglobulin (β_2m) and ICP47 are indicated. Molecular mass standards (M_r) were 69K, 46K, 30K, 14.3K and 10K (from top). The fluorographs in **a** and **b** were exposed for 48 h and 36 h, respectively. In several experiments, reprecipitated TAP2 displayed a slightly retarded mobility in SDS gels compared with TAP2 coprecipitated by anti-ICP47-C antibody. A small amount of the class I heavy chain (lane 12 in Fig. 3b) was reprecipitated by anti-TAP2 antibody. This might reflect reassociation of class I heavy chain and TAP2 during the reprecipitation following SDS denaturation. Arrowheads in **a** indicate unidentified proteins coprecipitated by anti-TAP1 antibodies. Because some of these proteins are also found in anti-TAP2 immunoprecipitates (not shown), they might be specifically associated with TAP complexes. Arrowheads in **b** indicate unidentified proteins immunoprecipitated by anti-ICP47-C antibody which are not related to ICP47, as they are also detected in anti-ICP47-C immunoprecipitates obtained from HtTa cells not transfected with ICP47. HtTa⁹, the parental HeLa cell of the stable ICP-O20 transfectant, was used as negative control in **a** and **b** because non-induced ICP-O20 cells express small amounts of ICP47 in the presence of tetracycline.

METHODS. ICP-O20 cells (2×10^6) were cultured for 2 days in the presence of $2,500 \text{ U ml}^{-1}$ IFN- γ before labelling with 0.15 mCi ml^{-1} ^{35}S -methionine (Tran ^{35}S -label, ICN) overnight in methionine-deficient medium followed by 2 h labelling with 0.5 mCi ml^{-1} in methionine-deficient medium. Cells were lysed in lysis buffer containing either 1% NP40 or 1% digitonin¹⁴. Immunoprecipitations, SDS-PAGE and fluorography have been described¹¹. For reprecipitation, the immunoprecipitated material was denatured in 1%



SDS at 100°C for 10 min and, after dilution to 0.05% SDS with 1% NP40 in PBS, again immunoprecipitated with the respective antibodies.

suggests that the viral protein might be acting by interacting directly with the peptide transporters. Previous immunofluorescence data demonstrated that ICP47 is primarily cytosolic, and there was no evidence of staining of the ER membrane, the known location of the TAP¹², or of binding to microsomes⁸. When we stained ICP-O20 cells cultured in the absence of tetracycline with anti-ICP47-C, an antiserum raised in rabbits against the carboxy-terminal 20 residues of ICP47, we also observed a staining pattern that was coincident with a cytosolic protein (Fig. 2c). By contrast, when ICP47 expression was reduced by adding tetracycline (Fig. 2d), the immunofluorescence pattern observed for ICP47 was indistinguishable from that of TAP stained with a monoclonal antibody against the TAP2 subunit (Fig. 2f). This perinuclear ER-staining pattern was also found with an antiserum specific for proteins resident in the ER¹³ (Fig. 2e). Moreover, transfected HLA-B27 was found exclusively in the same compartment after ICP47 had been induced (Fig. 2b), in contrast to both surface and intracellular staining in non-induced cells (Fig. 2a). The difference observed between anti-ICP47 staining patterns at different induction levels suggests

that, at low intracellular concentrations, ICP47 is enriched at the ER membrane. Upon overexpression such binding to ER is saturated, and most ICP47 remains unbound in the cytosol, which is therefore strongly stained, obscuring staining of the ER.

We examined whether localization of ICP47 in the ER was the result of ICP47 binding to TAP. A monoclonal antibody against TAP1 was used to immunoprecipitate TAP1 from NP40 or digitonin lysates of ICP-O20 cells or untransfected HtTa cells. the parental HeLa cells of ICP-O20 transfectants. As shown in Fig. 3a, we observed that TAP2 coprecipitated with TAP1 from HtTa or ICP-O20 cell lysates, as indicated by reprecipitation using a monoclonal antibody specific for TAP2 (Fig. 3a, lane 5). Consistent with previous reports that TAP forms a complex with empty class I molecules^{14,15}, we found that anti-TAP1 immunoprecipitates obtained from digitonin lysates contain class I heavy chain and β_2 -microglobulin (Fig. 3a, lanes 6 and 7). Moreover, in addition to these proteins, anti-TAP1 immunoprecipitates obtained from both NP40 and digitonin lysates of ICP-O20 cells contain a protein corresponding in size to the

predicted molecular mass of ICP47 (ref. 8) (Fig. 3a, lanes 3 and 4) not present in HtTa cell lysates (Fig. 3a, lanes 1 and 2). The identity of this coprecipitated protein with ICP47 was confirmed by using anti-ICP47-C antibodies (Fig. 3a, lane 8). Binding of ICP47 to TAP was also observed in the reciprocal experiment in which we immunoprecipitated ICP47 with anti-ICP47-C antibodies (Fig. 3b, lanes 1 and 11) from ICP-O20 cell lysates, followed by reprecipitation with anti-TAP2 (Fig. 3b, lanes 2 and 12) and antibodies specific to class I (Fig. 3b, lanes 3, 4, 13 and 14) as well as anti-ICP47-N, an antiserum raised in rabbits against the amino-terminal 20 residues of ICP47 (Fig. 3b, lanes 5 and 15). Again we observed that ICP47 coprecipitated with TAP in NP40 or digitonin lysates prepared from ICP-O20 cells (Fig. 3b, lanes 1 and 11) but not from HtTa cells (Fig. 3b, lanes 6 and 16). Furthermore, both class I heavy chain and β 2-microglobulin were present in anti-ICP47 immunoprecipitates obtained from digitonin lysates of ICP-O20 cells, indicating that ICP47 does not disrupt the class I/TAP complex. Murine TAP molecules did not coprecipitate with ICP47 (not shown), consistent with the hypothesis that ICP47 binding is relatively specific for human TAP. Taken together with our immunofluorescence observations (Fig. 2), these results indicate that ICP47 specifically binds to human TAP, an interaction that interferes with the peptide translocation mechanism. Our findings that ICP47 binds to and inhibits TAP activity provides the first evidence

that the peptide translocating activity of TAP can be modulated *in vivo* by molecules other than ATP. □

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Purification of an MCM-containing complex as a component of the DNA replication licensing system

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REPLICATION licensing factor (RLF) ensures that eukaryotic chromosomal DNA is replicated exactly once in each cell cycle^{1–4}. On exit from metaphase, RLF is activated and binds to or modifies chromatin. This modification (the 'licence') is required for subsequent DNA replication; the licence is also inactivated in the process of replication. Active RLF is not imported into the nucleus, so further DNA replication cannot occur until the DNA is relicensed by passage through mitosis. We have developed an assay to purify RLF from *Xenopus* eggs⁴. Activity resolves into two components, RLF-M and RLF-B, both of which are required for licensing. RLF-M has been purified to apparent homogeneity: it consists of three polypeptides, one of which is a *Xenopus* homologue of the yeast MCM3 protein. *Xenopus* Mcm3 associates with chromatin in G1 and is removed during replication, consistent with its being a component of the RLF system.

RLF is activated during mitosis, and activation can be blocked by protein kinase inhibitors such as 6-dimethylaminopurine (6-DMAP)^{4–6}. We have designed an assay for RLF, using 6-DMAP to generate *Xenopus* egg extracts devoid of RLF activity. In the 'licensing reaction', chromatin is incubated for 15 min with RLF candidate fractions, and is then added to extracts treated with 6-DMAP (consequently lacking RLF), for the 'replication

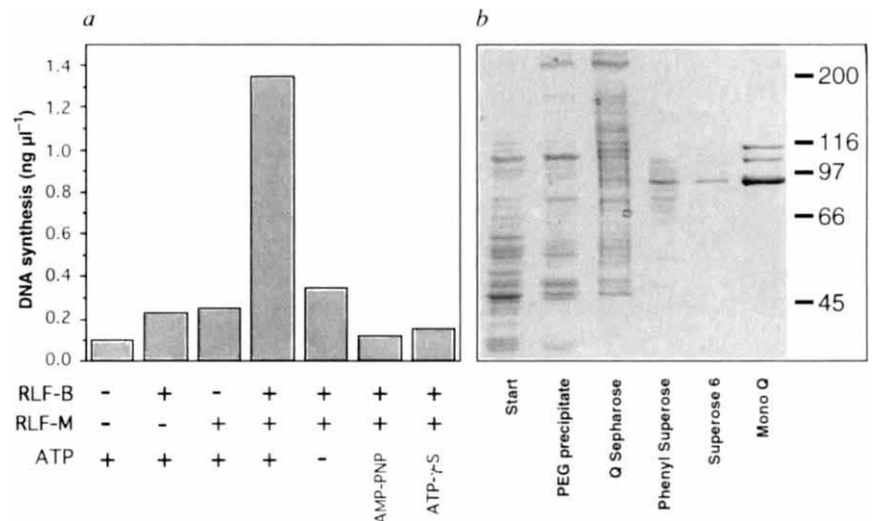
reaction'. Only if the licensing reaction contains active RLF is the DNA replicated in this procedure⁴. Initial fractionation by polyethylene glycol (PEG) precipitation separated RLF into two components: RLF-M, an Mcm-containing fraction, and RLF-B, which apparently contains a DNA-binding activity. Both RLF-M and RLF-B were essential for licensing (Fig. 1a). The presence of hydrolysable ATP was also required in the licensing reaction. The RLF-M fraction was purified over four further chromatographic steps, and assayed in the presence of crude RLF-B (Fig. 1b). In the last step (Fig. 2a), RLF-M activity coeluted with three prominent polypeptides of relative molecular mass (M_r) 115K, 106K and 92K in a ratio of approximately 1:1:4. However the stoichiometry between these bands varied between different preparations. These three polypeptides together accounted for >95% of the protein in the most purified fractions. On gel filtration they comigrated with an apparent M_r 490K to 660K ('Superose 6' fraction in Fig. 1b) and >300K by glycerol gradient sedimentation (data not shown), suggesting that they associate into a complex with high molecular mass.

Because Mcm (mini-chromosome maintenance) proteins are potential components of RLF^{7,8}, we blotted fractions from the final chromatographic step with antibodies raised against recombinant *Xenopus* Mcm3 (Fig. 2b: for details of the antibody see ref. 9). The 106K polypeptide was recognized by this antibody (Fig. 2b, lane 19), and by two antibodies raised against conserved peptide sequences in the Mcm family (S. Donovan and J. F. X. Diffley, unpublished data). The 92K polypeptide also crossreacted with these Mcm peptide antibodies (data not shown), suggesting that it is also a member of the Mcm family. The 115K polypeptide was strongly recognized by antibodies raised against BM28, a human Mcm2 homologue¹⁰ (data not shown). To confirm the involvement of Mcm3 in the licensing reaction, extract was immunodepleted with anti-Mcm3 antibody, reducing total Mcm3 protein by 92% (Fig. 2c). Immunodepletion caused a marked reduction in licensing activity which was efficiently rescued with crude RLF-M fractions, and less efficiently with purified RLF-M (Fig. 2d). More severe depletion of Mcm3 resulted in a complete abolition of licensing activity (data not shown). Immunodepletion with this antibody blocked DNA replication in *Xenopus* extracts⁹, and immunodepletion

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FIG. 1 Fractionation of RLF-M. *a*, PEG precipitation of *Xenopus* extracts separated activity into two components: RLF-B and RLF-M. Licensing assays with combinations of these fractions and 1 mM ATP, AMP-PNP or ATP- γ -S are shown. *b*, After PEG precipitation, RLF-M was purified by Q Sepharose, Phenyl Superose, Superose 6 and Mono Q fractionation. Activity was monitored in the presence of crude RLF-B and ATP. Fractions at each stage were electrophoresed on 7.5% polyacrylamide gels and stained with Coomassie blue. Molecular mass markers are indicated to the right.

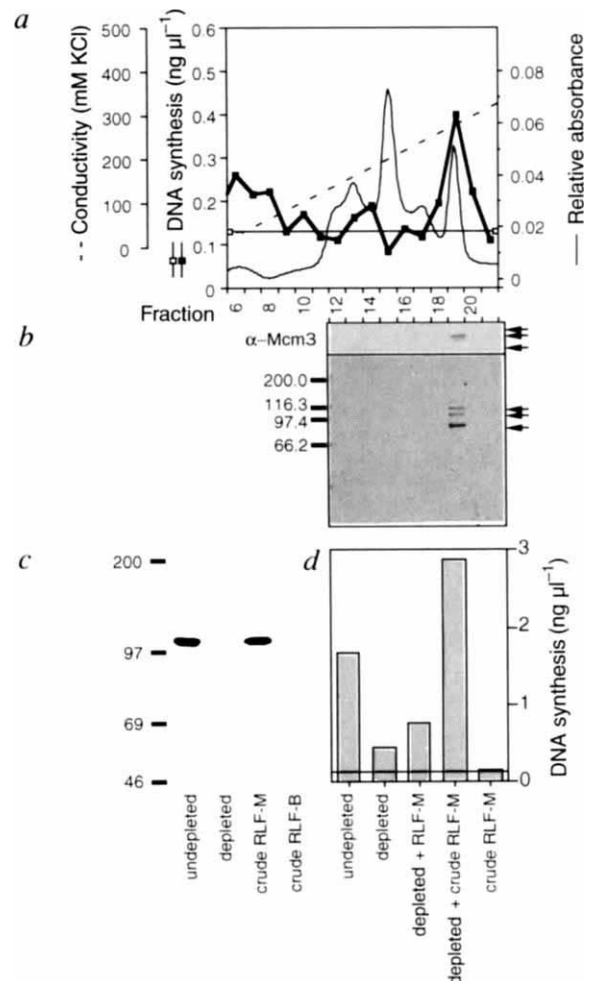
METHODS. RLF assay: The licensing assay was as described⁴. The 6-DMAP chromatin was prepared by incubation in 6-DMAP extract for 15 min, isolated by centrifugation⁴ and frozen; it was incubated with assay fractions for 15 min at 23 °C (licensing reaction). The 6-DMAP-extract containing [α -³²P]dATP was then added and incubated for a further 90 min (replication reaction). DNA synthesis was assayed by TCA precipitation. For RLF-M purification assays, each licensing reaction contained crude RLF-B and 2.5 mM Mg-ATP. RLF-M purification: Activated *Xenopus* egg extracts prepared as described⁴ to the spin-crush step were diluted with 4 vol LFB1 + 50 mM KCl (LFB1 contained 40 mM HEPES-KOH, 20 mM K₂HPO₄/KH₂PO₄, 2 mM MgCl₂, 1 mM EGTA, 2 mM DTT, 10% sucrose, 1 μ g ml⁻¹ leupeptin, pepstatin and aprotinin, 0.5 mM PMSF, pH 8.0) and centrifuged at 50,000 g, 20 min, 4 °C, in a swing-out rotor (*b*, Start). Sequential PEG 6000 precipitation generated RLF-B (0 to 4.25% PEG) and RLF-M (4.25 to 9% PEG) (*b*, PEG precipitate). RLF-M was adsorbed onto



Q Sepharose (Pharmacia) in LFB1 + 100 mM KCl and eluted in LFB1 + 325 mM KCl (*b*, Q Sepharose). Activity was applied to a 1 ml Phenyl Superose column (Pharmacia) in LFB1 + 1 M KCl and eluted over a 4 ml gradient to LFB0 (LFB1 with 20 mM HEPES minus phosphate). Active fractions (*b*, Phenyl Superose) were precipitated with 15% PEG and applied to a SMART system Superose 6 gel filtration column (Pharmacia) in LFB1 + 75 mM KCl. Active fractions (*M_r* ~ 490K to 660K) (*b*, Superose 6) were fractionated on a SMART system Mono Q column (Pharmacia) (*b*, Mono Q); see Fig. 2.

FIG. 2 Purified RLF-M contains Mcm3 protein. *a*, *b*, The Superose 6 RLF-M was fractionated on a Mono Q column. Individual fractions were assayed for RLF activity (*a*, filled squares), electrophoresed on polyacrylamide gels and stained with Coomassie (*b*, lower panel) or western blotted and probed with polyclonal antibody specific for *Xenopus* Mcm3 (*b*, upper panel). *a*, Also the RLF-B alone background signal (open squares), the ultraviolet absorbance at 280 nm (solid line) and the salt elution gradient (broken line). *c*, Extract was immunodepleted with Mcm3 antibody and western blotted alongside crude PEG fractions of RLF-M and RLF-B. *d*, Undepleted and depleted extract was used in the RLF assay. Depleted extract was reconstituted either with purified RLF-M or with the crude PEG fraction of RLF-M. Activity of crude RLF-M and the background with buffer alone (horizontal line) are also shown.

METHODS. Pooled Superose 6 fractions were applied to a SMART system Mono Q column (Pharmacia) equilibrated in LFB1 + 75 mM KCl and developed over a 20 column volume gradient to LFB1 + 500 mM KCl. Fractions were precipitated with 20% PEG and resuspended in LFB1 + 50 mM KCl + 2.5 mM Mg-ATP before assay and PAGE. The western blot was probed with an affinity-purified rabbit antiserum raised against a recombinant fusion protein containing the carboxy terminus (amino acids 423 to 799) of the *Xenopus* Mcm3 protein (see ref. 9 for details of the antibody and the immunodepletion technique). Western blot detection used the ECL system (Amersham).



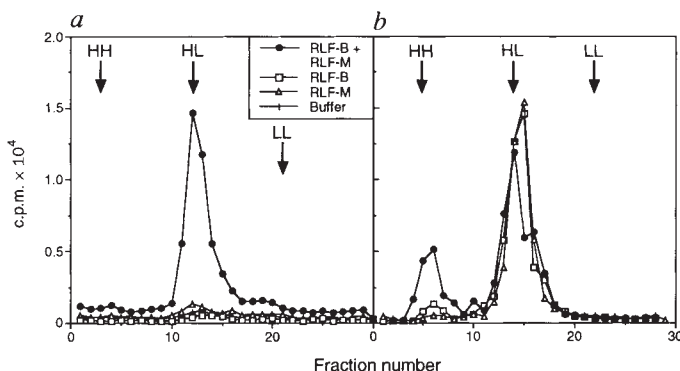


FIG. 3 RFL-M and RFL-B are required for every additional round of semiconservative DNA replication. *a*, Unreplicated chromatin was licensed with either buffer alone, purified RFL-M alone, crude RFL-B alone, or a combination of purified RFL-M and crude RFL-B, and

with a different anti-Mcm3 antibody also blocked licensing²⁸. The relatively inefficient rescue of Mcm3-depleted extracts by purified RFL-M suggests that other Mcm3-associated proteins are also required for efficient licensing.

The involvement of Mcm proteins in the licensing reaction is consistent with existing information about this family^{7,8}. Mcm homologues are widely distributed among eukaryotes, and are essential for DNA replication^{7,10-21}. MCM2, MCM3 and MCM5 (also called CDC46) are all required for DNA replication in budding yeast, and show genetic interaction with replication origins^{7,11-13,16}. In mammalian cells, DNA replication was blocked by antibodies to BM28, a human Mcm2 homologue¹⁰, and to murine P1, an Mcm3 homologue^{14,20}.

We next investigated the role of purified RFL-M in DNA replication. Density substitution experiments demonstrated that both purified RFL-M and crude RFL-B were required to license a single round of semiconservative replication in extracts treated with 6-DMAP (Fig. 3*a*). To prevent re-replication of DNA in a single cell cycle, the licence is inactivated as replication occurs,

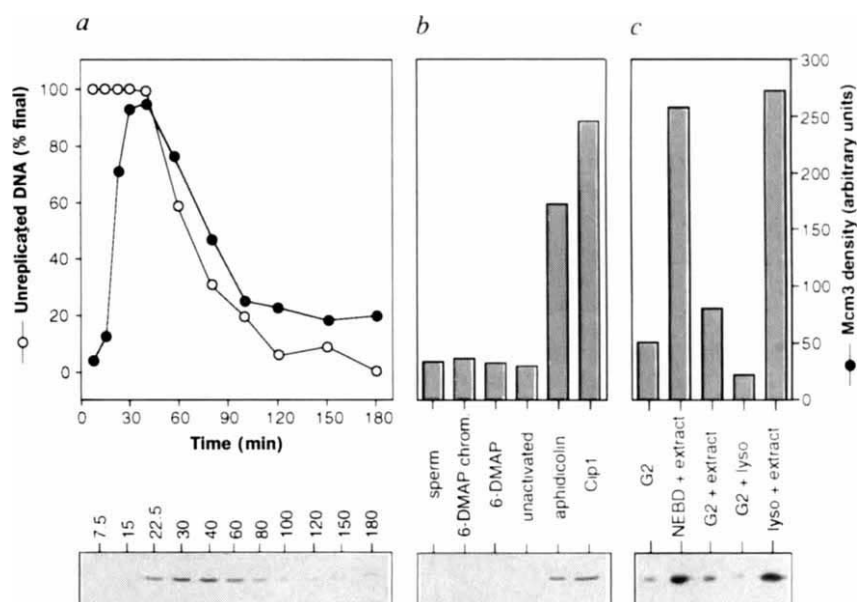
incubated in 6-DMAP extracts supplemented with BrdUTP and [³²P]dATP. After 90 min DNA was isolated and fractionated on neutral CsCl equilibrium density gradients. The expected densities of unsubstituted (LL), fully substituted (HH) and half-substituted (HL) double-stranded DNA are indicated. *b*, As *a*, except that the template DNA had previously been replicated once in normal *Xenopus* extract in the presence of BrdUTP and [³H]dATP. Replicated nuclei were isolated, permeabilized with lysolecithin and then treated with purified RFL-M and/or crude RFL-B before incubation for 90 min in 6-DMAP extract supplemented with BrdUTP and [³²P]dATP.

METHODS. BrdUTP density substitution was as described²⁷. To prepare replicated nuclei, 300 ng demembranated *Xenopus* sperm DNA was incubated for 3 h at 22 °C in 100 µl *Xenopus* extract supplemented with cycloheximide, [³H]dATP and BrdUTP. G2 nuclei were isolated, treated with 100 µg ml⁻¹ lysolecithin for 5 min at room temperature and re-isolated^{1,4}. After incubation with purified RFL-M and/or crude RFL-B, chromatin was incubated for 3 h in 6-DMAP extract supplemented with [³²P]dATP and BrdUTP. Density shift of the ³H-labelled DNA showed that ~25% of the replicated DNA replicated a second time following treatment with both RFL-M and RFL-B.

so that RLF must interact with DNA before each round of replication¹. Figure 3*b* shows that this feature applies to purified RFL-M. Replicated nuclei labelled with BrdUTP were isolated, permeabilized with lysolecithin¹ and incubated with purified RFL-M, crude RFL-B or a mixture of the two, before transfer to 6-DMAP extract containing [³²P]dATP and BrdUTP. Only when replicated chromatin was exposed to both RFL-M and RFL-B did significant re-replication occur (Fig. 3*b*, filled circles). The extent of re-replication seen in the presence of both RFL-M and RFL-B (approximately 25% of available template) is typical for the amount of re-replication observed following lysolecithin permeabilization¹.

Xenopus Mcm3 became associated with chromatin over a period of 10 to 20 min (Fig. 4*a*, filled circles), during which time licensing occurs⁴. When DNA replication started after about 40 min, the amount of chromatin-bound Mcm3 declined in direct proportion to the amount of unreplicated DNA remaining in the extract (Fig. 4*a*, open circles), which suggests that RFL-M dissociates from chromatin as it replicates. To demonstrate

FIG. 4 Mcm3 is associated only with licensed chromatin. *a*, Demembranated *Xenopus* sperm nuclei were added to metaphase-arrested *Xenopus* extracts released into interphase with CaCl₂. At different times after release, chromatin was isolated and immunoblotted with Mcm3 antibody (lower). The Mcm3 signal was quantified and expressed in arbitrary units (upper, filled circles). In parallel, DNA synthesis was measured by [³²P]dATP incorporation and expressed in terms of unreplicated DNA remaining in the extract (upper, open circles). *b*, In parallel with *a*, extract was supplemented with 3 mM 6-DMAP, 30 µg ml⁻¹ aphidicolin or 150 nM Cip1, or was not released with CaCl₂ (unactivated extract). After 3 h chromatin was isolated and blotted for Mcm3. DNA synthesis under these conditions was less than 5%. Demembranated *Xenopus* sperm nuclei and 6-DMAP chromatin were also blotted. *c*, G2 nuclei replicated in *Xenopus* extract supplemented with cycloheximide were isolated and transferred to interphase extract or to metaphase arrested extract for 15 min (causing nuclear envelope breakdown, NEBD) before release with CaCl₂; alternatively G2 nuclei were treated with lysolecithin (lyso.) and added to interphase extract. Extracts were supplemented with 30 µg ml⁻¹ aphidicolin to prevent re-replication, and incubated for 90 min at 23 °C. Chromatin was then isolated and immunoblotted for Mcm3 as above. **METHODS.** Chromatin was isolated by resuspending in NIB (50 mM KCl, 50 mM HEPES KOH, 5 mM MgCl₂, 2 mM β-mercaptoethanol, 0.5 mM spermidine 3HCl, 0.15 mM spermine 4HCl, 1 µg ml⁻¹ each leupeptin, aprotinin and pepstatin, pH 7.6) containing 0.1% NP-40, and centrifug-



ing at 6000 g in a swing-out rotor through a 100 µl cushion of NIB containing 0.1% NP-40 and 15% sucrose. Pelleted material was electrophoresed on 7.5% polyacrylamide gels, western blotted and probed with MCM3 antibody using ECL detection. Isolation of intact G2 nuclei was as described^{1,4}. Glutathione S-transferase (GST)-tagged human Cip1 was as previously described²².

that dissociation was a direct consequence of replication, various inhibitors were added. When the initiation of DNA replication was blocked by p21^{Cip1} at concentrations sufficient to inhibit Cdk2:cyclin E^{22,23}, no loss of chromatin-bound Mcm3 was observed (Fig. 4b). Only a slight reduction of chromatin-bound Mcm3 was seen in extracts containing aphidicolin, which allows initiation to occur but blocks replication fork elongation^{4,22,24}. In extracts treated with 6-DMAP or arrested in metaphase, to prevent licensing⁴, Mcm3 did not associate with chromatin (Fig. 4b). These results are consistent with experiments in mouse cells, in which subnuclear redistribution of Mcm3 was observed during S phase²⁰. Similarly in yeast, MCM2, MCM3 and MCM5/CDC46 proteins are exclusively nuclear in late mitosis and G1, but disappear from the nucleus at the onset of DNA replication^{7,16}.

For DNA to be re-licensed for further replication, the nuclear envelope must be transiently permeabilized^{1,4}. G2 nuclei were isolated and used to assess the ability of Mcm3 to rebind chromatin following various treatments (Fig. 4c). Intact nuclei transferred directly into interphase extract showed only a slight increase in chromatin-bound Mcm3 (Fig. 4c, G2+extract). However, a large increase in chromatin-bound Mcm3 was observed when the nuclear envelope was permeabilized before addition to interphase extract, by either inducing mitotic nuclear envelope breakdown (Fig. 4c, G2+NEBD) or lysolecithin treatment (Fig. 4c, lyso+extract). These results show that re-association of Mcm3 with replicated chromatin is dependent on nuclear envelope permeabilization (as normally occurs during mitosis) and correlates with the re-licensing of DNA.

Our results can explain why DNA is replicated no more than once in each cell cycle. However, because re-replication of DNA in a single cell cycle is potentially so detrimental, there may be additional mechanisms to prevent it. Licensing of DNA by RLF-M and RLF-B correlates with the association between Mcm3 and chromatin early in the cell cycle. Mcm3, which is a component of RLF-M required for DNA replication, is removed from the chromatin as replication proceeds. DNA cannot re-replicate until it is re-licensed by active RLF-M and RLF-B, and this does not occur until the nuclear envelope has been permeabilized during mitosis. The role of nuclear envelope permeabilization is currently unclear. Although RLF activity does not cross the nuclear envelope in *Xenopus*^{1-5,25} or *Drosophila*²⁶, Mcm proteins are nuclear throughout the mammalian cell cycle^{10,14,20}, and Mcm3 is imported into intact nuclei in *Xenopus* extracts⁹. One possibility is that active RLF-B is required for RLF-M to associate with chromatin, and that RLF-B is incapable of crossing the nuclear envelope. This could be demonstrated by purification and characterization of the RLF-B fraction. □

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MCM3 complex required for cell cycle regulation of DNA replication in vertebrate cells

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An intact nuclear membrane restricts DNA replication to only one round in each cell cycle, apparently by excluding an essential replication-licensing factor throughout interphase¹⁻⁵. A family of related yeast replication proteins, MCM2, 3 and 5 (also called, after cell-division cycle, CDC46), resemble licensing factor, entering the nucleus only during mitosis⁶⁻⁸. We have cloned a *Xenopus* homologue of MCM3 (XMCM3) and raised antibodies against expressed protein. Immunodepletion of *Xenopus* egg extracts removes a complex of MCM2, 3 and 5 homologues and inhibits replication of *Xenopus* sperm nuclei or permeable G2 HeLa nuclei. However, G1 HeLa nuclei still replicate efficiently. Mock-depleted extracts replicate all three templates. XMCM3 accumulates in nuclei before replication but anti-XMCM3 staining decreases during replication. These results can explain why replicated nuclei are unable to reinitiate replication in a single cell cycle.

A *Xenopus laevis* homologue of the yeast MCM3 gene was cloned from an egg complementary DNA library as described in Fig. 1. It encodes a protein of 807 amino acids that is 48% and 71% identical to yeast⁹ and human¹⁰ MCM3, respectively. The sequence of XMCM3 is available in the GenBank database (accession number U26057).

Amino acids 438 to 625 were expressed in *Escherichia coli* and used to raise rabbit polyclonal antibodies which were purified from antisera of four rabbits by affinity chromatography against immobilized protein. Figure 1a shows that anti-XMCM3 antibodies specifically immunoprecipitate four proteins from *Xenopus* egg extracts which appear to form a complex. One of these proteins reacts with the original anti-XMCM3 antibody on Western blots (Fig. 1b, lane 3). Two others react specifically with antibodies against human MCM2 (ref. 11) (Fig. 1b, lane 2) or *Drosophila* MCM5/CDC46 (Fig. 1b, lane 4). The remaining band sometimes resolves into a doublet. It appears to be another member of the MCM family because it reacts with an antibody against a conserved domain present throughout the MCM2/3/5 protein family¹² (Fig. 1b, lane 1). These proteins precipitate together with antibodies against XMCM3 raised in four separate rabbits (Fig. 1a and data not shown), but not with any of the control antibodies tested (Fig. 1a and data not shown). Further evidence exists for a complex of MCM proteins in *Xenopus*^{13,19}, humans¹⁴ and *Drosophila* (T. Su and P. H. O'Farrell, personal communication).

Affinity-purified anti-XMCM3 antibodies were used separately to immunodeplete the MCM complex from *Xenopus* egg extract. Control antibodies were used identically for mock depletion. Figure 1b shows that XMCM3 is removed completely from depleted extract (lane 5 versus lane 6). Figure 2 shows that

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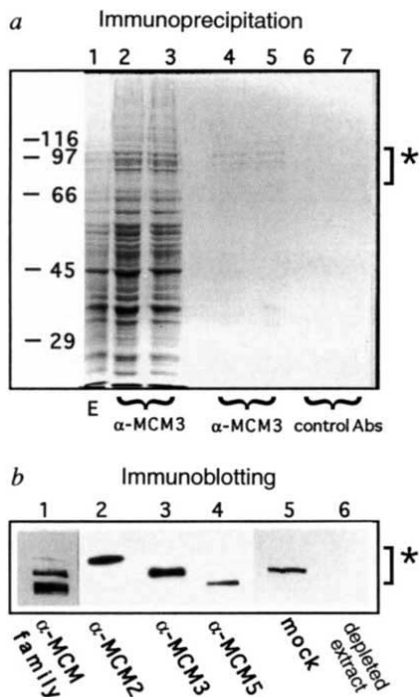


FIG. 1 Antibodies against XMCM3 remove a complex containing MCM 2, 3 and 5. **a**, Two anti-XMCM3 antibodies (antibodies 1 and 2) immunoprecipitate the same four proteins from *Xenopus* egg extracts (asterisk) (lanes 4 and 5). Rabbit pre-immune and rabbit anti-rat IgG do not precipitate specific proteins (lanes 6 and 7). Serial immunoprecipitations with anti-XMCM3 antibodies 1 and 2 do not change the protein profile (lanes 2 and 3) compared with undepleted extract (E, lane 1). **b**, Anti-XMCM3 immunoprecipitates contain proteins that react with antibodies against *Xenopus* XMCM3, human BM28 (ref. 11) (a homologue of MCM2; gift from I. Todorov and S. E. Kearsey), and *Drosophila* CDC46 (MCM5; gift from T. Su and P. H. O'Farrell), indicating that the complex contains homologues of MCM2, MCM3 and MCM5 (lanes 2, 3 and 4). All four proteins are recognized by an antibody against a conserved domain present throughout the MCM2/3/5 protein family¹² (lane 1) (gift from R. Burkhardt). Two immunoprecipitations with anti-XMCM3 completely remove XMCM3 from extracts (**b**, lanes 5 and 6). METHODS. Degenerate primers were designed against conserved regions in MCM3 protein sequences^{10,21} using MacVector (IBI). The nucleotide sequences of the primers are: 5'-AT(A/C/T)CA(C/T)GA-(A/G)GT(A/T/C/G)ATGGA(A/G)CA-3' and 5'-CAT(T/C/G/A)AC(C/T)TG (A/G)TT(G/A)TC-3', corresponding to amino-acid sequences IHEVMEQ (positions 423–429) and DNQVM (positions 794–798), respectively. From a *Xenopus* egg cDNA library²², a 1.1-kb PCR product was cloned and used to screen an oocyte λ gt10 cDNA library^{23,24}. The 5' end was obtained by polymerase chain reaction (PCR). Antibodies were raised against expressed recombinant protein, using pGEX-3X (Pharmacia) and pQE32 (Qiagen) in *E. coli*²⁵. Four glutathione S-transferase (GST)-XMCM3 fusion protein preparations were injected into rabbits. His-tagged fusion protein crosslinked to CNBr-activated Sepharose (Pharmacia) in the presence of 1% SDS was used to affinity purify the antibodies²⁶. Egg supernatants²⁷ were incubated at 4 °C for 45 min with antibodies pre-bound to protein-A Sepharose beads (Pharmacia). Beads were recovered by spinning, and 7% polyacrylamide gels were Coomassie stained or immunoblotted using ECL detection (Amersham).

depletion with three different anti-XMCM3 antibodies, but not three control antibodies, abolishes replication of *Xenopus* sperm nuclei as measured by incorporation of α^{32} P-dATP (Fig. 2a) and incorporation of biotin-dUTP (Fig. 2b). In contrast, nuclear decondensation and selective import of nuclear proteins are not inhibited (Fig. 2b and data not shown). We conclude that remo-

val of the MCM protein complex specifically inhibits DNA replication. This is consistent with the phenotypes of MCM2, 3 and 5 mutations in *S. cerevisiae*^{15,16} and with results of anti-MCM3 or MCM2 antibody injections into cultured mammalian cells^{11,17}. Figure 2a (track 8) and c confirms this conclusion. Replication is restored to an extract depleted by one anti-MCM3

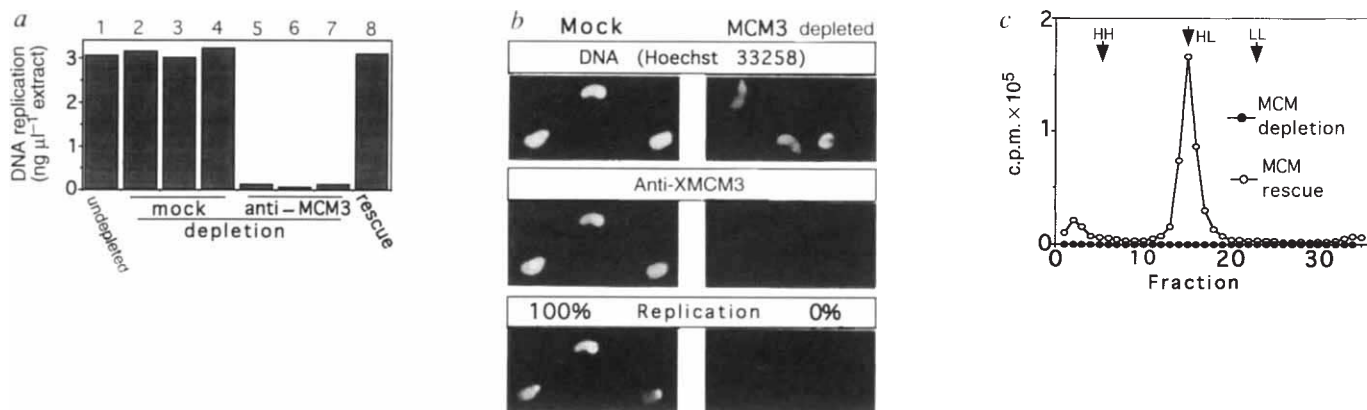


FIG. 2 Immunodepletion by monospecific anti-XMCM3 antibodies inhibits DNA replication in *Xenopus* egg extracts. **a**, Sperm nuclei replicate efficiently in undepleted extract or extract mock immunodepleted with control antibodies (track 2, rabbit pre-immune; track 3, rabbit anti-rat IgG; track 4, rabbit anti-goat IgG). In contrast, immunodepletion of the MCM protein complex with any one of three anti-XMCM3 antibodies abolishes replication (tracks 5–7). Re-addition of MCM complex immunopurified by a fourth anti-XMCM3 antibody rescues replication in extract depleted by antibody 1. **b**, Sperm chromatin in mock depleted egg extracts stain positive by immunofluorescence for XMCM3 and incorporate biotin-dUTP into DNA efficiently. Depletion of the MCM protein complex results in normal nuclear formation but loss of XMCM3 immunostaining and inhibition of replication. **c**, Density substitution confirms that re-addition of purified MCM complex rescues replication in a depleted extract, as in track 8 of Fig. 2a. METHODS. High- or low-speed egg supernatants were immunodepleted

(antibodies 1, 2 or 3) or mock depleted as described in Fig. 1. MCM complex was eluted from antibody 4 with 0.8 M NaCl, 50% ethylene glycol¹⁹ in extract buffer¹ and dialysed against 10% sucrose in extract buffer. Sperm chromatin, egg membranes²⁸, cycloheximide and energy regenerator were added to immunodepleted and mock immunodepleted extracts at 23 °C. **a**, Incubations were supplemented with α^{32} P-dATP (DuPont NEN) for 5 h, and incorporation into DNA determined²⁷. **b**, Alternatively biotin-dUTP (Boehringer Mannheim) was added. After 40 min, nuclei were spun onto coverslips, fixed in 4% paraformaldehyde and stained with Hoechst 33258 to show total DNA, with Texas Red streptavidin (Amersham) to detect biotin-dUTP incorporation and with rabbit anti-XMCM3 (antibody 1) followed by fluorescein-conjugated anti-rabbit antibody (Amersham) to show localization of XMCM3. More than 100 nuclei were scored for biotin incorporation. **c**, BrdUTP (0.4 mM, Sigma) was added with α^{32} P-dATP. DNA was extracted and fractionated on CsCl density gradients²⁷.

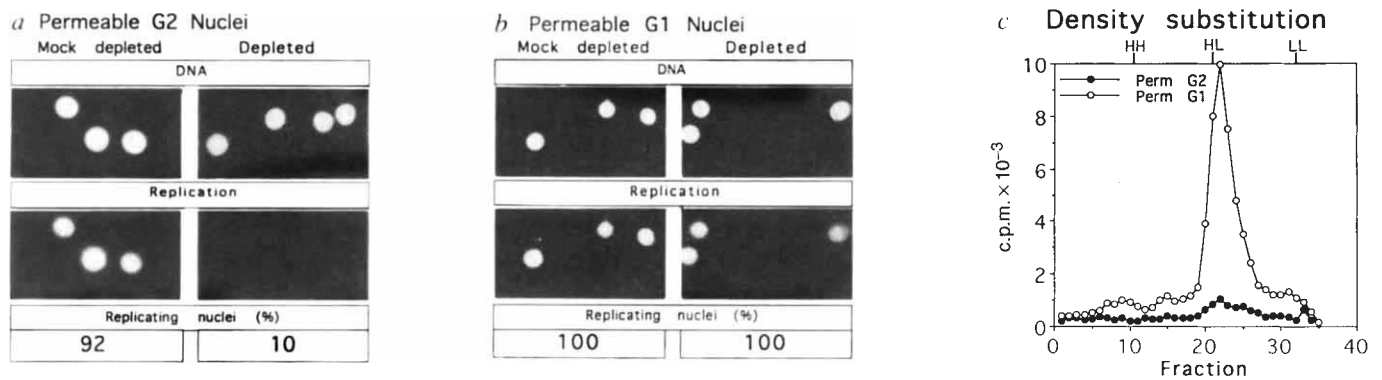


FIG. 3 An MCM protein complex is required for permeable HeLa G2 nuclei but not for permeable G1 nuclei to replicate in egg extract. Mock depletion of egg extract does not affect replication of either template. **a**, **b**, Replication was monitored by biotin-dUTP incorporation. **c**, Density substitution revealed that permeable G1 nuclei show a single round of semiconservative replication in extracts depleted of the MCM protein complex, whereas permeable G2 nuclei show no

incorporation in such extracts, even though they replicate in complete extract.

METHODS. Permeable HeLa nuclei^{2,3} were incubated for 5 h in immunodepleted (antibody 1 or 2) or mock-depleted egg extracts and harvested as described in Fig. 2 (**a**, **b**). More than 100 nuclei were scored for biotin incorporation. **c**, Density substitution was performed as in Fig. 2c.

antibody when the complex purified from a different anti-MCM3 antibody is added back. These experiments do not show that a single MCM protein such as MCM3 is essential, rather that the MCM complex itself is required.

Intact human G1 nuclei replicate in *Xenopus* egg extract but intact G2 nuclei do not^{2,3}, indicating that the extract can distinguish replicated from unreplicated templates; this is consistent with cell fusion results¹⁸. However, when the nuclear envelope is permeabilized, G2 nuclei become able to replicate in egg extract^{2,3}. To investigate whether MCM proteins are involved in distinguishing G1 from G2 nuclei by licensing a single round of replication in each cell cycle, we tested the dependence of permeable G1 and G2 nuclei on the exogenous XMCM3 complex for replication. Figure 3a shows that permeable G2 HeLa nuclei replicate in mock-depleted egg extract but that immunodepletion with anti-XMCM3 antibodies abolishes their replication. In con-

trast, Fig. 3b shows that G1 HeLa nuclei do not require *Xenopus* egg XMCM3 for replication. Unlike G2 nuclei, replication of G1 nuclei is undiminished by complete immunodepletion of XMCM3. Figure 3c shows that the DNA synthesized by G1 HeLa nuclei in *Xenopus* egg extracts depleted of XMCM3 represents complete semiconservative replication, and emphasizes the clear difference between G1 and G2 nuclei in their dependence on the MCM protein complex.

These results can be explained if the MCM protein complex is essential for DNA replication and if G1, but not G2, nuclei contain an active MCM complex already, and are thus 'licensed' for a round of replication. Figure 4a supports this conclusion by showing that XMCM3 immunofluorescence rises sharply before replication in sperm nuclei incubated in egg extract, but is lost during replication. This decrease in XMCM3 immunofluorescence during replication is consistent with observations made with

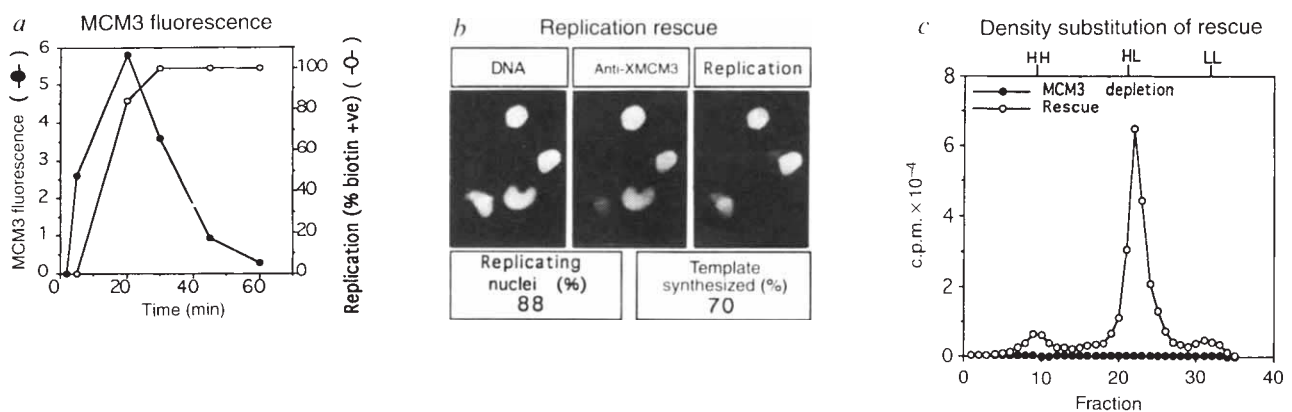


FIG. 4 **a**, XMCM3 binds sperm chromatin before initiation but decreases in immunostaining during replication. **b**, XMCM3 can cross an intact nuclear membrane and rescue replication of sperm nuclei depleted of the MCM protein complex. **c**, Depleted nuclei that show no incorporation by density substitution become competent to replicate after transfer to an MCM-containing, undepleted extract resulting in a single round of semiconservative replication.

METHODS. **a**, Sperm chromatin was incubated with biotin-dUTP in undepleted extract, collected at various times and stained as described in Fig. 2. For each time point 10 or more random nuclei were quantified for biotin incorporation. XMCM3 immunofluorescence was quantified by comparing fluorescent intensities collected at identical settings on the scanning laser confocal microscope (BioRad MRC600). Data

representing total pixel intensity across the equatorial plane of each nucleus was estimated either with Comos (data not shown) or with NIH Image software. Results from both sets of analyses were similar. **b**, As described in Fig. 2, sperm chromatin was incubated for 40 min in egg extract depleted using anti-XMCM3 (antibody 1 or 2). Nuclei were transferred to fresh egg extract supplemented with either biotin-dUTP or [α^{32} P]dATP and 0.4 mM BrdUTP. Integrity of the nuclear envelope was monitored at all times using fluorescein-labelled nucleoplasmin core fragment²⁰ (data not shown). XMCM3 immunofluorescence and replication were monitored by immunostaining after 30 min incubation in fresh egg extract (**b**) or by density substitution after 5 h in fresh egg extract supplemented with [α^{32} P]dATP and 0.4 mM BrdUTP (**c**).

yeast^{6,8}, detergent-washed synchronized mammalian cells¹⁷ and *Xenopus* egg extracts^{13,19}. It is confirmed by immunoblotting¹⁷ (data not shown). Therefore the ability of G1 but not G2 nuclei to replicate without XMCM3 correlates with the presence of MCM3 before replication and its loss from chromatin during replication¹⁷. However, if the MCM proteins are the full explanation of the replication licensing phenomenon¹, then they should be excluded by the nuclear envelope. XMCM3 can cross an intact nuclear membrane before replication initiates (Fig. 4b), and it restores replication of an intact nucleus assembled in its absence (Fig. 4b, c). The integrity of the nuclear membrane throughout the entire experiment was verified by exclusion from the nuclei of a fluorescein-conjugated protein that lacks a nuclear localization signal (nucleoplasmic core)²⁰ (data not shown). Therefore XMCM3 and presumably the other proteins of the MCM complex can cross the nuclear membrane before initiation, which is consistent with MCM3 entering the nucleus during G1 of somatic cells¹⁷.

This observation contrasts with the regulated nuclear entry of MCM3 in yeast^{6,9} and with the nuclear membrane exclusion predicted for 'licensing factor'¹. So why is permeabilization of the nuclear membrane required to allow a replicated nucleus to replicate again¹⁻³? We shall show elsewhere that nuclear envelope permeabilization is required for MCM3 to bind to chromatin rather than to enter the nucleus (M.A.M., C.Y.K., A.D.M. and R.A.L., manuscript in preparation). Presumably a second component is required to load the MCM complex onto chromatin, and this second factor is excluded by the nuclear membrane. A good candidate for this second factor is fraction RLF-B (described in ref. 13).

We describe here an MCM complex which is essential for DNA replication, but lost or inactivated during replication. These properties can explain the synchronized cell fusion results¹⁸ and the *in vitro* results using *Xenopus* egg extracts¹⁻⁵.

Thus they also explain why replicated nuclei are unable to reinitiate DNA replication until they have bound an active MCM complex during mitosis. □

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Structure of tubulin at 6.5 Å and location of the taxol-binding site

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TUBULIN, the major component of microtubules, is a heterodimer of two chains, α and β , both of relative molecular mass 50,000 (M , 50K) and with 40–50% identity. The isotopic variety² and conformational flexibility of tubulin have so far made it impossible to obtain crystals for X-ray work³. Structural knowledge of tubulin has been limited to about 20 Å from X-ray diffraction of oriented microtubules⁴, and from electron microscopy of microtubules and zinc-induced crystalline sheets in negative stain^{5,6}. The sheets consist of protofilaments similar to those in microtubules but associated in an antiparallel arrangement⁷, and their two-dimensional character is ideal for high-resolution electron microscopy^{8,9}. Here we present a three-dimensional reconstruction of tubulin to 6.5 Å resolution, obtained by electron crystallography of zinc-induced two-dimensional crystals of the protein. The α - and β -subunits appear topologically similar, in agreement with their sequence homology¹⁰. Several features can be defined in terms of secondary structure. An apparent α -helical portion, adjacent to both inter-

dimer and inter-protofilament contacts, is tentatively attributed to a segment near the carboxy terminus of the protein. We can assign the α - and β -subunits on the basis of projection studies of the binding of taxol*, which show one taxol site per tubulin heterodimer, in agreement with the known stoichiometry of taxol in microtubules¹¹. These studies indicate that taxol affects the interaction between protofilaments; to our knowledge, this is the first time that a ligand-binding site has been visualized in the tubulin molecule.

Taxol enhances microtubule polymerization¹², inhibits cell replication by preventing spindle dynamics¹³, and has anti-tumour properties¹⁴. It stabilizes both microtubules¹⁵ and zinc-induced tubulin crystals¹⁶ against low-temperature depolymerization and ageing. We collected images from untitled tubulin crystals embedded in tannic acid with and without taxol to obtain a difference map representing the bound drug. We used tubulin isotopically purified for the β_{II} isotype ($\alpha\beta_{II}$ -tubulin)¹⁷, as the crystals show better order than undifferentiated phosphocellulose-purified tubulin (PC-tubulin). Figure 1a, b shows projection maps to 3.8 Å of crystals with and without bound taxol, respectively. Because contrast in these maps is generated by variations in the internal density of the protein, molecular boundaries are not clearly visible. To indicate the monomer and dimer outlines, we superimposed a contour map obtained from negatively stained samples of PC-tubulin crystals⁹. The difference map in Fig. 1c shows a position of high density repeating every two tubulin subunits along the protofilament, near the inter-protofilament contact, but far from the monomer–monomer or dimer–dimer contacts. We interpret this density as the binding site of the drug, corresponding to taxol itself, and/or to a small

* The word 'Taxol' is a registered trademark of Bristol-Myers Squibb, which offers the term paclitaxel instead.

FIG. 1 Projection density map at 3.8 Å resolution for tannin-embedded tubulin crystals *a*, without taxol and *b*, with bound taxol. Higher density is white. Zonal scaling of the Fourier amplitudes was used to minimize the differences between the two data sets. Superimposed on each projection is a contour map obtained from negatively stained samples of PC-tubulin crystals without taxol, merged to a common phase origin using a third data set obtained with glucose-embedded samples that were rinsed in uranyl acetate⁹ (the contour level was chosen to emphasize dimer boundaries). *c*, Difference map obtained by subtracting the projection densities of crystals without taxol from those with taxol (contrast enhanced three-fold). *d*, Significance map of the difference calculated using the Student's *t*-test for a confidence level of 99.5% (one-sided)⁴⁰.

Significant positive and negative differences are displayed in white and black, respectively.

METHODS. Microtubules were isolated from bovine cerebra and subjected to phosphocellulose chromatography to obtain PC-tubulin⁴¹. To obtain $\alpha\beta_{II}$ -tubulin, PC-tubulin was passed through immunoaffinity columns containing anti- β_{III} and anti- β_{IV} . Protein solutions (6.8 mg ml⁻¹), in 0.1 M MES, 0.5 mM MgCl₂, 1 mM EGTA, 0.1 mM EDTA, 1 mM β -mercaptoethanol, 1 mM GTP, pH 6.4, were diluted by half in an incubation buffer containing 80 mM MES, 200 mM NaCl, 4 mM GTP, 1.5 mM MgSO₄, 1.5 mM ZnSO₄, 0.04 mg ml⁻¹ pepstatin, 20% glycerol, pH 5.3. The final pH was 5.9–6.0. The sample was incubated for 24 h at 32 °C. After that time equimolar taxol was added to some of the sample, and grids were prepared within 1 h by the lens method⁴², using 3% tannin solution in water, pH 5.9. Grids were stored in liquid nitrogen until used.

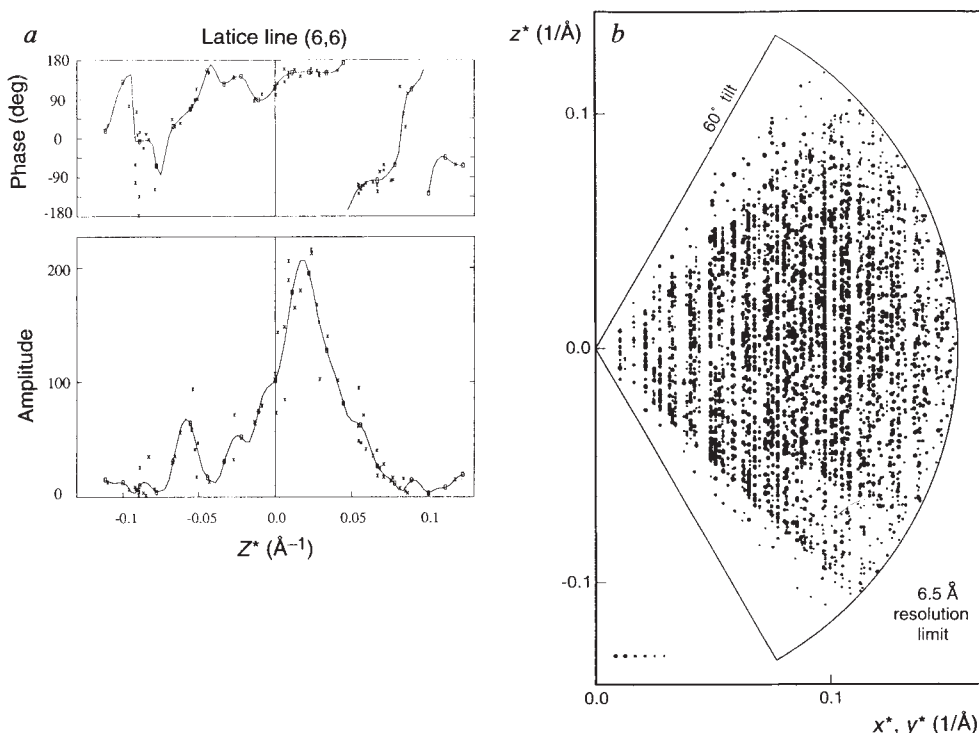
section of the protein that may become more ordered upon drug binding. It is unlikely that this density corresponds to a conformational change far from the binding site, as that would require that taxol does not induce or contribute to any density change at the actual site (taxol, M_r 0.85K). The reliability of the difference map is shown in the significance map in Fig. 1*d*. Regions of significant difference correspond to the taxol binding site and

Images were recorded on a JEOL 4000 electron microscope with the specimen at -170 °C, using low-dose techniques and spot-scan imaging⁴³, at an original magnification of $\times 60,000$. We used a range of underfocus values to avoid the systematic exclusion of any reflection. Selected regions in the images (typically 100 $\times$ 100 unit cells) were digitized and subjected to several cycles of unbending to correct for small lattice distortions⁴⁴. Images were corrected for contrast transfer function (CTF) by observation of the CTF zeros in plots of the signal to noise ratio at each reciprocal lattice point. In the initial merging step we used previous projection data from PC-tubulin⁸ as a reference. A new reference was created with the $\alpha\beta_{II}$ -tubulin images, and then used to refine the CTF correction. CTF-corrected files were merged again and a new reference created. The final merge included beam tilt corrections.

to some density shift occurring near the site, between adjacent protofilaments. As photolabelling studies indicate that taxol binds preferentially to the β -subunit^{18,19}, our projection study identifies the β monomer.

The location of the taxol binding site near the interprotofilament contacts is consistent with the preferential binding of the drug to the polymerized conformation of tubulin^{20,21}, and with

FIG. 2 *a*, Amplitudes and phases to 6.5 Å resolution along lattice line (6,6) (resolution for $z^* = 0$ is 10.1 Å). Curves were fitted by least-squares to the experimental amplitudes and phases (crosses), and then sampled at (90 Å)⁻¹ (circles) to generate the three-dimensional set of structure factors. *b*, Azimuthal projection of the three-dimensional data set, showing data quality and sampling. The size of the dots represents the signal to noise ratio; the smallest spot corresponds to a ratio of 2, the largest to a ratio of 7 or higher. Purification and crystallization of PC-tubulin were as previously described⁹. Dynamic focus correction⁴⁵ made unnecessary, at this resolution, any further focus compensation within the images of tilted specimens. Ten untilted images were brought to a common phase origin using previous projection data⁹. After generation of a new reference, 6 images at 30° tilt, 30 images at 45° tilt, and 4 at 55–60° were merged. A new reference including all the images was used to refine the crystal tilt for each image. $P2_1$ symmetry was imposed throughout merging. For the reconstruction, we used a total of 2,281 unique structure factors. This reconstruction does not include electron-diffraction data. Because the image amplitudes show a relatively slow falloff up to 6 Å resolution, we consider the effect of this exclusion minimal.



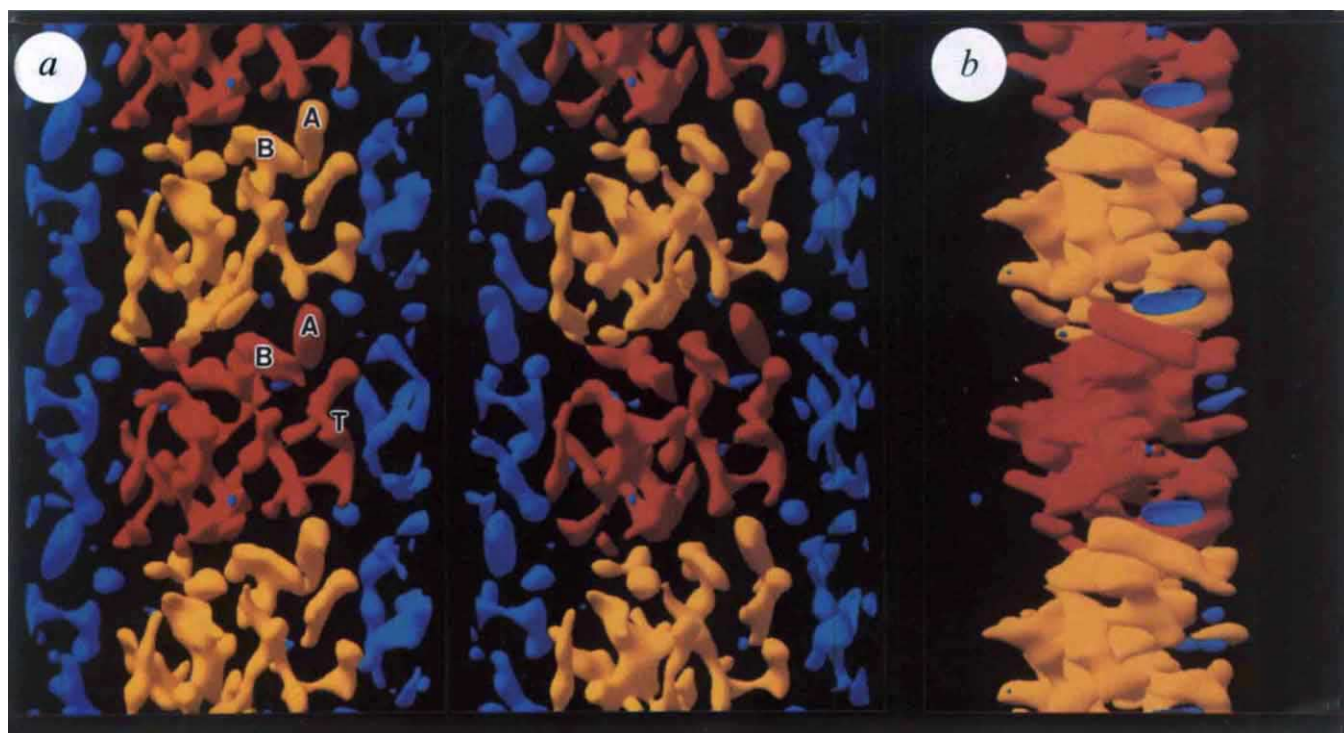


FIG. 3 *a*, Stereo pair of tubulin in a zinc-induced sheet embedded in tannic acid. In the central protofilament the α -subunits are yellow and the β -subunits red. Adjacent protofilaments are blue. Features tentatively identified in the text as α -helix and β -sheet are indicated in the central dimer by the letters A and B, respectively. T marks the

approximate position of the taxol binding site. *b*, Tangential view of a single protofilament. The rugged profile on the left side indicates that it corresponds to the inside of the microtubule wall (surface facing the viewer in *a*).

the reduction in the number of protofilaments in microtubules polymerized in the presence of taxol²². It favours those hypotheses that relate the action of taxol to the stabilization or strengthening of lateral interactions between protofilaments^{23,24}, and opposes those that localize its action at the longitudinal contacts between dimers²⁵. That taxol binds to and stabilizes zinc-induced sheets, where the inter-protofilament contact topology is different from that of microtubules, supports the notion that taxol binding is restricted to one monomer. Our results, however, cannot rule out a bridging effect of taxol involving the binding across the protofilament interface²³.

For the three-dimensional reconstruction we used 50 images of PC-tubulin crystals stabilized with taxol. The resolution of the map was limited by the falloff in data in high-tilt images perpendicular to the tilt axis. Figure 2*a* shows the amplitudes and phases for one lattice line, as an example of lattice-line sampling. A representation of data quality and completeness is shown in Fig. 2*b* as a function of tilt angle and resolution. The average phase residual was $24 \pm 6^\circ$, on merging data from all images. In the stereo pair of the 6.5-Å reconstruction in Fig. 3*a*, the monomer boundaries, indicated by colours, have been tentatively assigned based on contours from negatively stained samples⁹, and on the apparent connectivity of the features in the map. 'T' indicates the approximate position of taxol.

The reconstruction shows several well-resolved secondary structure domains. A cylindrical density (marked A) 25 Å long, near both the lateral and longitudinal interfaces between subunits, is identified as an α -helix by comparison with similar densities observed at this resolution for structures now solved by electron crystallography^{26–29}. A flattened slab of density in the interior of both subunits (marked B) corresponds to a region that in projection shows stripes of density roughly perpendicular to the protofilament axis (Fig. 1*a, b*), and so was predicted to be β -sheet⁸. Figure 3*b* is a tangential view of a single protofilament. One side is flat whereas the other appears ribbed. X-

ray diffraction and electron microscopy studies of microtubules have shown a predominance of helical grooves on the inside surface and longitudinal protofilament grooves on the outside^{4,7,30}. This property, which is particularly clear in images of partly opened microtubules observed by freeze-fracture³¹, is consistent with the orientation of the reconstruction in Fig. 3*b*.

Predictions of secondary structure for α - and β -tubulins (S.G.W., E.N., D. Gratzinger and K.H.D., in preparation) suggest only two α -helices, common to both subunits and both at the C terminus (α , 382–402; β , 371–391; and α , 418–436; β , 407–426), of sufficient length to accommodate the density of helix A. The tentative assignment of A to a region near the C-terminus is in agreement with chemical cross linking and limited proteolysis studies, which located the C-terminal domain of the α -subunit at the inter-dimer contacts³². Proteolysis studies suggested a local unfolding of an α -helix at around Arg 390 in β -tubulin, following the binding of colchicine³³. The position of such an α -helix at the inter-protofilament contacts would explain the fact that colchicine does not bind to microtubules³⁴, where the α -helix is buried, but binds tightly to vinblastine-induced coils³⁵, where the lateral interaction sites are still accessible.

This study provides the most detailed tubulin structure to date. Although many questions remain unanswered, several structural features can be interpreted in terms of biological function. At this resolution we cannot resolve the nucleotide or clearly define a domain-organization that would indicate the possible binding site. However, photolabelling evidence suggests that both taxol and GTP bind near the N terminus of β -tubulin^{36,37}, and the proximity of these sites is consistent with taxol being the only known ligand that can induce microtubule polymerization in the absence of the nucleotide γ -phosphate^{11,24,38,39}. Further interpretation of the tubulin structure clearly requires higher resolution. Recent developments in our embedding technique have dramatically improved the 'flatness' of our samples¹⁶. It is now possible to collect images and

diffraction patterns at high tilts which will soon allow us to obtain a three-dimensional density map at sufficient resolution to trace the tubulin peptide chain. □

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Ice-binding structure and mechanism of an antifreeze protein from winter flounder

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ANTIFREEZE proteins provide fish with protection against the freezing effect of polar environments by binding to ice surfaces and inhibiting growth of ice crystals. We present the X-ray crystal structure at 1.5 Å resolution of a lone α -helical antifreeze protein from winter flounder, which provides a detailed look at its ice-

binding features. These consist of four repeated ice-binding motifs, the side chains of which are inherently rigid or restrained by pairwise side-chain interactions to form a flat binding surface. Elaborate amino- and carboxy-terminal cap structures are also present, which explain the protein's rich α -helical content in solution. We propose an ice-binding model that accounts for the binding specificity of the antifreeze protein along the $\langle 0112 \rangle$ axes of the $\{2021\}$ ice planes¹.

Winter flounder antifreeze proteins (AFPs) are members of the α -helical class of AFPs (Fig. 1a). The crystal structure of the HPLC6 isoform was determined at 4 °C and –180 °C by molecular replacement using an idealized helix as a search model (methods and refinement statistics are summarized in Table 1). Four unique environments were sampled by the AFP in the two crystal structures, which assists in distinguishing relevant structure from crystal-induced artefacts. The AFP molecule is completely α -helical, with the exception of the last peptide unit which adopts a 3_1 -helix conformation. Figure 1b shows that AFP molecule (2) is straight whereas molecule (1) is gently bowed; this difference in curvature has no discernible effect on backbone hydrogen-bond lengths and probably reflects the flex-

TABLE 1 Data collection parameters and refinement statistics

Space group $P2_1$, cell parameters			
4 °C	$a = 38.17$ (7) Å	$b = 37.01$ (6) Å	$c = 21.84$ (2) Å
	$\beta = 101.1$ (1)°		
–180 °C	$a = 37.41$ (3) Å	$b = 36.56$ (4) Å	$c = 21.35$ (2) Å
	$\beta = 103.34$ (1)°		
Data collection statistics			
	4 °C		–180 °C
Resolution	1.7–40 Å		1.5–40 Å
No. of observed reflections ($>1\sigma$)	16,614		28,251
No. of unique reflections ($>1\sigma$)	5,505		8,329
Completeness	81.7%		89.2%
R_{merge}^*	4.4%		5.4%
Refinement parameters			
No. of molecules per asymmetric unit	2		2
No. of protein atoms	454		454
No. of water molecules	39		130
Resolution	1.7–8.0 Å		1.5–8.0 Å
No. of reflections ($>1\sigma$)	5,429		8,218
$R_{\text{factor}}/R_{\text{free}}^{\dagger}$	17.9/22.6%		17.7/19.6%
Deviation from ideal bond distances	0.014 Å		0.013 Å
Deviation from ideal bond angles	2.2°		1.8°
Disorder regions	Asn 27, molecule 1		None

Protein crystals were grown at 4 °C as described¹⁸. Diffraction data were collected at 4 °C and at –180 °C. The structure at 4 °C was solved by molecular replacement using a 37-mer of alanine in an α -helical conformation as a search model. The methods used will be described in detail elsewhere (manuscript in preparation). Conventional rotation and translation searches with both X-PLOR¹⁹ and MERLOT²⁰ failed to give a unique solution. A multi-parameter PC search (where PC is a Patterson or standard correlation coefficient obtained using the square of the normalized structure factors), was then attempted using X-PLOR. Search results were clustered and the top 1,620 solutions were rigid-body-minimized against R_{factor} . This was followed by positional refinement and simulated annealing, after which a correct solution was finally distinguishable from all other solutions. To resolve the minor rotational ambiguity that remained, the four threonine side chains of each AFP molecule were added at different positions and subsequent refinement identified the best placements. Following additional cycles of simulated annealing, inspection of $2|F_o| - |F_c|$ maps revealed the positions of the remaining side chains and several prominent water molecules. Solvent was then located using iterative cycles of water selection and refinement. Residual peak intensities, hydrogen-bonding contacts, steric contacts, refined B-factors and R_{factor} were taken into account in the selection procedure. An individual constrained isotropic B-factor refinement was performed on the final structure. The 4 °C structure without solvent was then used as a starting model for the –180 °C structure. Our AFP structure is similar to a previous crystal structure²¹ in crystal packing arrangement but differs in the direction and curvature of one of the two AFP molecules within the asymmetric unit.

* $R_{\text{merge}} = \sum |I_i - \langle I \rangle| / \sum I_i$, where I_i is the intensity of an individual reflection and $\langle I \rangle$ is the mean intensity of that reflection.

† $R_{\text{factor}} = \sum |F_o| - |F_c| / \sum |F_c|$, where F_o and F_c are the observed and calculated structure factor amplitudes respectively. Before crystallographic refinement, 10% of F_o were randomly selected and set aside for monitoring R_{free} .

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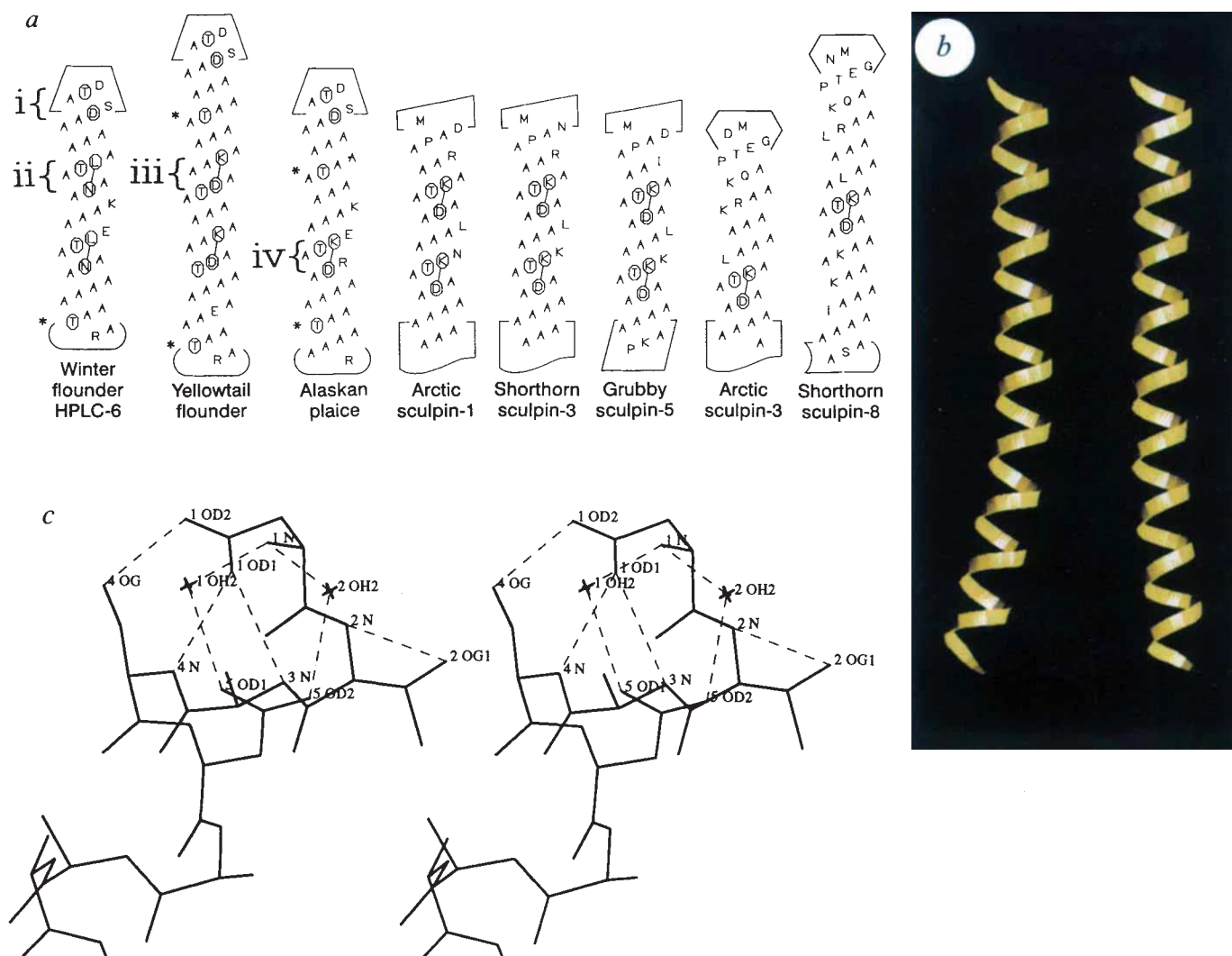


FIG. 1 *a*, Helical net representations of the alanine-rich α -helical AFPs from different fish. Representative IBMs are labelled i-iv. End-cap sequences predicted to have similar three-dimensional structures are shown as common shapes; IBMs are circled; residues that interact or are predicted to interact in order to stabilize ice-binding conformations are joined; incomplete IBMs are indicated with asterisks. AFP sequences are taken from refs 1 and 22. *b*, Ribbon diagrams of AFP molecules (1) and (2) (left and right respectively) demonstrate the flexibility of the protein's backbone structure. The curvature of each molecule is similar at 4 °C and -180 °C. AFP molecules are aligned with their amino termini up and ice-binding surfaces facing left. Backbone hydrogen-bond lengths are unaffected by the differences in backbone curvature; mean bond length for molecule (1) is 2.92 ± 0.07 Å and 2.94 ± 0.07 Å for molecule (2). Figures were generated using program O (ref. 23). *c*, Stereo drawings of the N- (top panel) and C-terminal cap structures. The helix axis, N- to C-terminal lies

in the plane of the page from top right to bottom left, respectively. Hydrogen bonds are represented by thin dashed lines. The two N-terminal cap-bound water molecules are labelled 1OH2 and 2OH2.

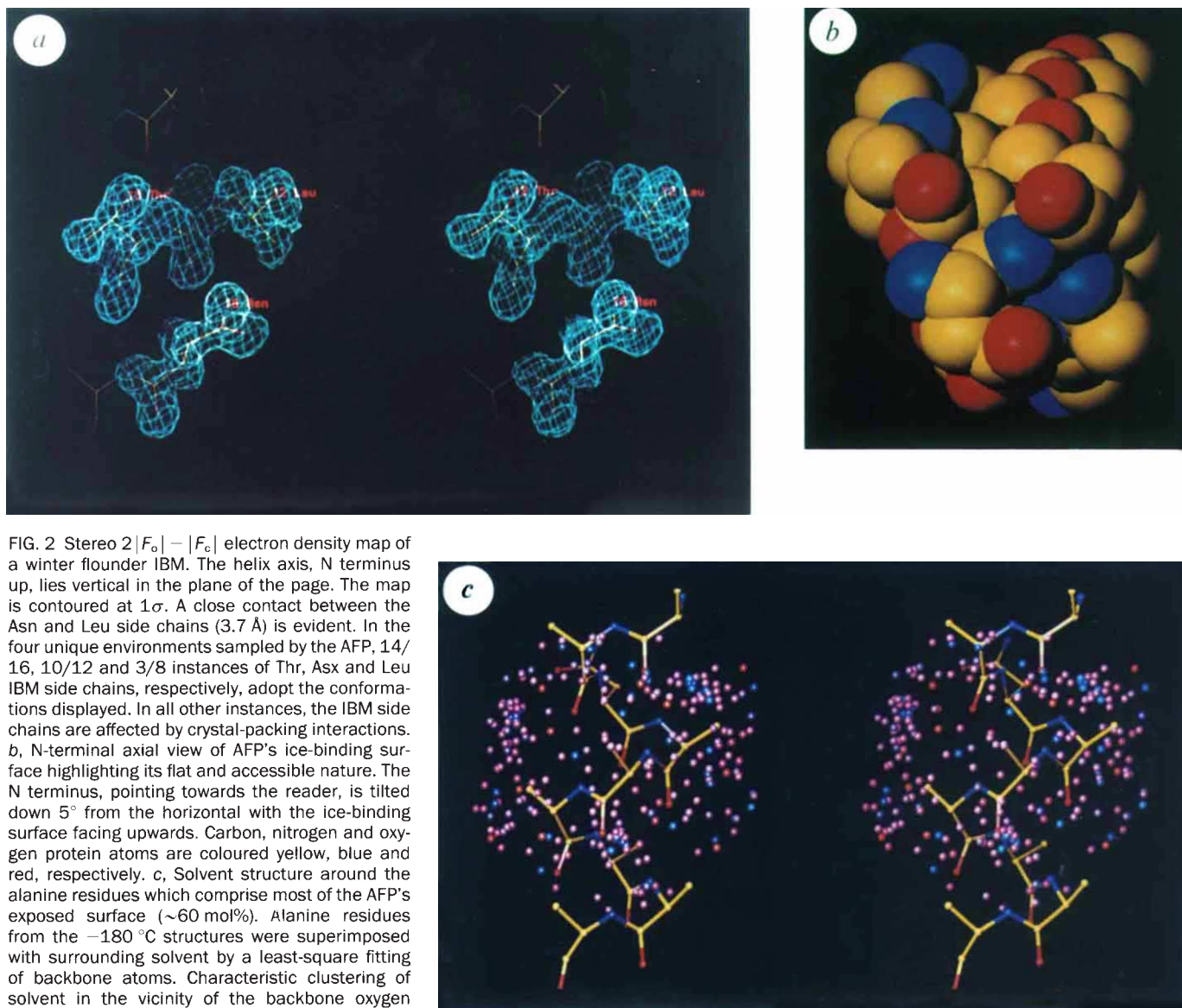


FIG. 2 Stereo $2|F_o| - |F_c|$ electron density map of a winter flounder IBM. The helix axis, N terminus up, lies vertical in the plane of the page. The map is contoured at 1σ . A close contact between the Asn and Leu side chains (3.7 Å) is evident. In the four unique environments sampled by the AFP, 14/16, 10/12 and 3/8 instances of Thr, Asx and Leu IBM side chains, respectively, adopt the conformations displayed. In all other instances, the IBM side chains are affected by crystal-packing interactions. *b*, N-terminal axial view of AFP's ice-binding surface highlighting its flat and accessible nature. The N terminus, pointing towards the reader, is tilted down 5° from the horizontal with the ice-binding surface facing upwards. Carbon, nitrogen and oxygen protein atoms are coloured yellow, blue and red, respectively. *c*, Solvent structure around the alanine residues which comprise most of the AFP's exposed surface (~ 60 mol%). Alanine residues from the -180°C structures were superimposed with surrounding solvent by a least-square fitting of backbone atoms. Characteristic clustering of solvent in the vicinity of the backbone oxygen atoms is evident⁹. No unusual solvent ordering is apparent. Protein atoms of the displayed α -helical segment (amino terminus up) are coloured as in *b*. Blue to red colouring of water molecules represents isotropic B-factor variations ranging from 7 to 55 Å (ref. 2). *d*, Solvent structure around IBM residues. IBMs from the -180°C struc-

tures with surrounding solvent were superimposed by a least-square fitting of backbone atoms. Protein atoms of the displayed IBM containing α -helical segment (amino terminus up) are coloured as in *b*. Water molecules are shown in red.

ibility of the protein. Of the 14 non-alanine side chains present in each AFP molecule, all appear to serve roles in either ice binding or helix stabilization.

Winter flounder AFPs are remarkably stable for lone α -helices in solution, with estimated helical contents approaching unity at 4°C (refs 2–6). Elaborate terminal cap structures^{7,8}, revealed in the crystal structure, are likely to contribute greatly to this stability (Fig. 1*c*). The N-terminal cap structure consists of an ordered network of eight hydrogen bonds involving the side chains of residues Asp 1, Thr 2, Ser 4, Asp 5 and two tightly bound water molecules. The hydrogen-bonding network effectively caps all four local backbone groups that lack intra-helical hydrogen-bonding partners. The apical positioning of the Asp 1 side chain within the cap structure also allows for a stabilizing interaction with the helix dipole, while the proximity of Asp 5 to the free N terminus may offset the latter's destabilizing charge. The C-terminal cap structure, characterized by a CaI helix distortion⁹, makes use of the side chain of Arg 37 and the amidated C terminus¹⁰ to form three hydrogen bonds. This leaves uncapped only one of four local backbone sites. The Arg 37 side

chain forms one capping hydrogen bond and is well positioned to interact favourably with the helix dipole. Amidation of the C terminus eliminates a destabilizing charge interaction with the helix dipole and replaces a hydrogen-bonding acceptor group with a donor that serves to cap the carbonyl group of Ala 35.

The AFP's ice-binding structure is composed of the repeat of four similar ice-binding motifs (IBMs) made up of the residues Thr/Asp or Thr/Asn/Leu (Fig. 1*a*). The last IBM is incomplete, having only a Thr residue. Figure 2*a, b* shows a front view of a single IBM and an axial view of the entire ice-binding surface, respectively. Unexpectedly, we find that IBM residues are rigidly constrained to conformations that produce an exceptionally flat ice-binding surface.

The β -branched threonine side chains are sterically constrained ($\chi_1 = -61 \pm 4^\circ$) by the α -helical conformation of the protein's backbone structure^{11,12}. The side chains of Asp 5, Asn 16 and Asn 27 adopt the same conformation ($\chi_1 = -75 \pm 3^\circ$, $\chi_2 = 13 \pm 13^\circ$) yet are restrained by different means. Asp 5 is held fixed by the capping interactions, while Asn 16 and Asn 27 form weak hydrogen bonds to the helix backbone (Fig. 2*a*). Close

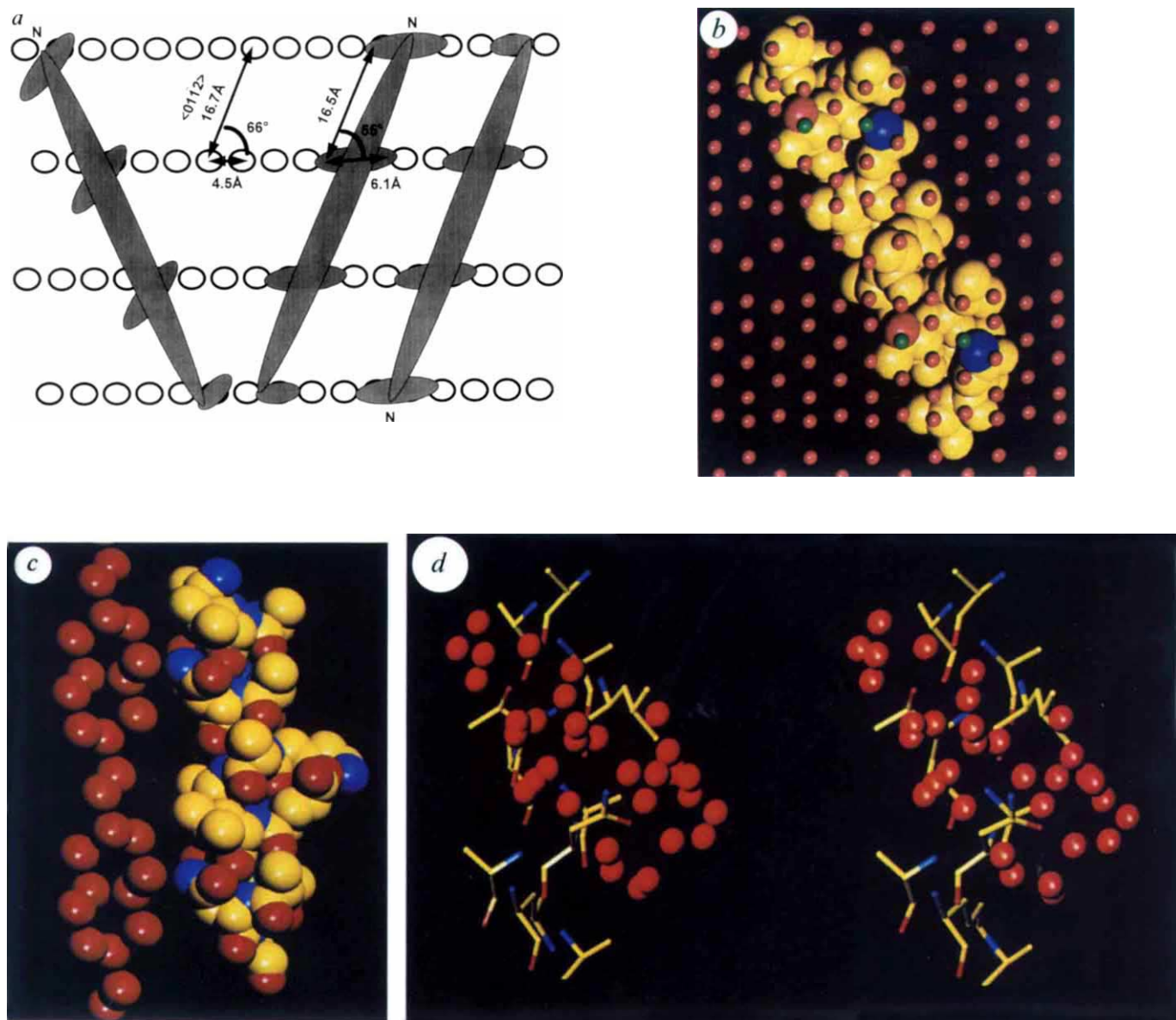


FIG. 3 Winter flounder AFP ice-binding model. *a*, Diagram to illustrate the complementarity between the AFP structure and the topology of the $\{2021\}$ ice plane to which the AFP binds. AFP molecules (shaded with N termini labelled), display the positioning and geometry of ice-binding groups relative to the helix axis. Water molecules that form the prominent ridges on the ice plane are shown as circles. The two AFP molecules on the right are aligned along the $\langle 0112 \rangle$ binding axis; the molecule on the left is aligned along the mirror-related axis. Because of the flatness of the AFP's ice-binding surface, our model indicates that the protein could bind along either direction of the $\langle 0112 \rangle$ axis, contrasting

with earlier predictions that the AFP orients itself in a single direction^{15,17}. The loss of complementarity could explain why the AFP does not bind along the mirror-related axis. *b*, Front, and *c*, side view of an AFP molecule (residues 9 to 28) docked along the $\langle 0112 \rangle$ binding axis of the $\{2021\}$ ice plane. The side views show the punctuated flatness of the ice-binding surface of the protein (right) and the ridge-and-valley topology of the ice plane (left). In *b*, protein atoms (with the exception of ice-binding groups) are shown in yellow and target molecules on the ice plane are shown in green.

van der Waals contacts between leucine and asparagine side chains suggest that the leucine residues play a part in stabilizing the asparagine conformations. This may account for the finding that substitution of leucine residues with alanine results in a 33% loss of protein function³. A comparison of the primary sequences of other α -helical AFPs shows that the pairing of IBM residues with $i, i+4$ spacing is a highly conserved feature (Fig. 1*a*). In other IBMs, aspartate is paired with lysine, except in the incomplete IBMs, where there is always a correlated absence of paired residues.

The restrained conformation of IBM side chains has a great influence on the accessibility of the AFP's ice-binding groups. As shown by the axial view of the ice-binding surface in Fig. 2*b*,

the OH, NH₂ and C=O groups of the Thr, Asn and Asp side chains, respectively, protrude slightly from a flat local surface composed with neighbouring apolar groups. This accessibility is critically maintained by the conserved use of alanine in the positions radially between the Thr and Asx ice-binding residues. The restrained conformation of IBM residues also results in a periodic spatial arrangement of the ice-binding groups. Equivalent groups within adjacent IBMs are separated by 16.5 ± 0.5 Å, whereas groups within a single IBM are separated by 6.3 ± 0.3 Å and form a 55° crossing angle with the helix axis.

Analysis of the solvent structure shows that the AFP has no enhanced ability to interact with water (Fig. 2*c*), but it does reveal a clue as to how the AFP might interact with ice. The

hydration pattern around the ice-binding side chains (Fig. 2d) shows no specific pattern but instead forms an elongated surface around the polar groups that is limited only by accessibility. This is consistent with the variation from ideal geometry observed for the hydrogen-bonding of water to the side chains of other proteins¹³. It is conceivable that less than perfect hydrogen-bonding geometry is employed in the AFP's selection of a target site on ice.

Current ice-binding models^{14,15} position binding groups to extend from the AFP's structure to occupy ice-lattice sites. In support of this, molecular modelling has identified a spatial match between the binding groups of the AFP and water molecules on the ice planes to which the AFP binds^{14,15}. This spacial match is also consistent with the AFP's $\langle 01\bar{1}2 \rangle$ axial alignment on the binding planes¹, although several features of our crystal structure appear to contradict this view. First, a sufficiently close spatial match between the AFP and ice is not apparent: whereas the average separation between equivalent ice-binding groups in adjacent IBMs closely matches the 16.7 Å $\langle 01\bar{1}2 \rangle$ axial repeat distance of water molecules on the binding plane, the closest match on ice found to fit the spacing of binding groups within an IBM is 4.5 Å. Second, the ice-binding groups do not protrude sufficiently from the AFP's ice-binding surface to clear sterically hindering groups; the C γ atoms of the threonine residues and the C β atoms of alanines 9, 20 and 31 would prevent binding groups from entering into ice even if a congruent lattice match were available. Third, the trigonal planar (SP²) coordination of asparagine and aspartate hydrogen-bonding groups differ from the tetrahedral (SP³) coordination of water molecules in ice.

In contrast, we envisage that the binding of AFP to ice is defined by a less stringent hydrogen-bonding criterion. This forms the basis of an ice-binding model (Fig. 3). A characteristic feature of the $\{20\bar{2}1\}$ binding plane is a ridge-and-valley topology^{15,17} which subtends the $\langle 01\bar{1}2 \rangle$ binding axis by 66°. This topology complements the AFP structure when the AFP is aligned along the binding axis. Considering that the hydrogen-bonding groups extend minimally from the AFP's flat ice-binding surface, water molecules on the ridges of the $\{20\bar{2}1\}$ ice plane are the most probable binding sites. The 4.5 Å spacing of water molecules along the ridges provides accessible hydrogen-bonding targets for both ice-binding groups within an IBM.

In conclusion, we believe that the relative flatness of the AFP's ice-binding surface and the rigidity of the side chains are critical to the AFP's ice-binding mechanism. The latter maintains the AFP in its ice-binding conformation while the former maximizes the accessibility of binding groups to an ice surface. Together these characteristics allow for the concerted formation of many hydrogen bonds between the AFP and ice, giving permanence to binding. Whereas the spatial arrangement of ice-binding residues contributes to the binding ability and specificity of the AFP, the underlying hydrogen-bonding interactions are likely to be more liberally defined than previously proposed^{14,15}. The finding that all of the AFP's structure appears to be dedicated to ice binding and helix stabilization strongly suggests that ice binding is the only essential characteristic required for the AFP to inhibit ice crystal growth. Our ice-binding model is amenable to testing and we are currently designing HPLC6 mutants for this purpose. A flat and rigid ice-binding surface, albeit with different spacial arrangements of ice-binding groups, may be a general feature of all AFPs. □

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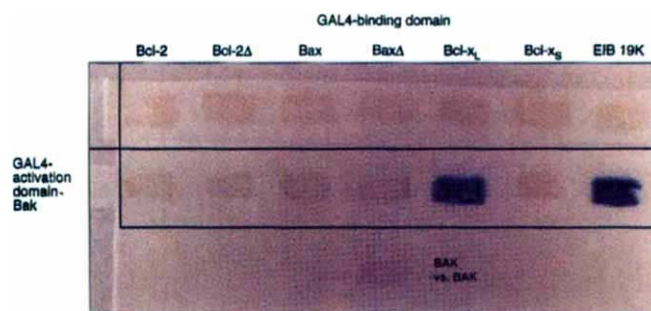
ERRATA

Cloning of a *bcl-2* homologue by interaction with adenovirus E1B 19K

Stuart N. Farrow, Julia H. M. White, Isabelle Martinou, Thomas Raven, Kwok-Tao Pun, Christine J. Grinham, Jean-Claude Martinou & Robin Brown

Nature **374**, 731–733 (1995)

FIGURE 4 of this Letter should have been reproduced in colour, as shown here. The last sentence of the caption is accordingly amended to read “A blue colour indicates interaction”. □



Silica aerogel films prepared at ambient pressure by using surface derivatization to induce reversible drying shrinkage

Sai S. Prakash, C. Jeffrey Brinker, Alan J. Hurd & Sudeep M. Rao

Nature **374**, 439–443 (1995)

PARTS *c* and *d* of Fig. 4A of this Letter were incorrectly labelled: part *c* should have been labelled as *d* and part *d* as *c*. □

Trademarks must not go generic

Neil White and Simon Cohen

The worldwide registration by a pharmaceutical company of the word *taxol* as a trademark suggests that procedures for registering such marks needs to be tightened.

THE news that the pharmaceutical company Bristol-Myers Squibb has registered the name *taxol* as a trademark in nearly 70 countries worldwide, potentially giving them the right to prevent anyone else from using the name, has given rise to widespread comment in the scientific community¹.

The company is seeking a monopoly on the name, even though it has been used by scientists throughout the world for many years to describe a chemical isolated from the pacific yew tree *Taxus brevifolia*. That product is still undergoing trials. But it appears to help in the treatment of cancer, and Bristol-Myers Squibb wishes to use the name to describe its own proprietary cancer treatments, benefiting from the reputation of a natural product.

The widespread comment that this has provoked suggests that many consider the company's move to be inappropriate². But the real question is how the registration come to be allowed by the trademark registries of the world in the first place. If examiners had been aware of the background to the application and made the required searches, they would surely have looked at it in a different light.

Trademark examiners carry out certain rudimentary searches before allowing a mark to be registered. For example, they look through the existing trademarks listed on the register and in other publicly available compilations, such as dictionaries. Apparently this search turned up nothing to challenge the company's trademark application. But a glance at any compendium of chemical terms, such as the *Merck Index*, would have revealed that *taxol* is used by scientists to describe the product isolated from *Taxus brevifolia*.

The immediate implication is that registries should adapt their searches to suit the type of proposed trademark being considered. Research on a chemical or pharmaceutical trademarks such as *taxol* should be carried out differently than one on ordinary words such as Budget (services involving the renting of vehicles and considered unregistrable), Mothercare (signifying equally care by mothers and care for mothers and held registrable) and, of course, *Nature*.

Trademark protection has traditionally given less attention than patents in com-

merce. But a genuine trademark, if properly policed, can be an invaluable asset. For example, whereas patent protection lasts for a limited period of time — in Europe, this is 20 years from filing — trademark protection can potentially last for ever, as long as renewal fees are paid and the mark does not become part of everyday vocabulary.

A trademark need not be a word but can be a distinctive shape or get-up (or even perhaps a smell or sound). Although under the previous law, the UK House of Lords refused to register the distinctive shape of the Coca-Cola bottle, in contrast they allowed the registration of SmithKline Beecham's multi-colour pharmaceutical capsules. Under the Trade Marks Act 1994, it is expected that distinctive shapes and get-ups will become easier to register.

The significance of brand names and trademarks is widely recognised by accountants, and the asset value of trademarks is reflected in the balance sheets of companies. Examples such as Coke, Xerox and Band-aid each demonstrate how a trademark can become an extremely valuable property.

A trademark indicates that goods or services come from a particular source, distinguishing them from the comparable goods or services of competitors. But a word in everyday use cannot be captured and monopolised by registering it for use in this way. In a recent case, for example, the British Trade Marks Registry ruled that the company Budget Rent-A-Car Corporation should not be allowed to register marks consisting of the word budget for services that involve renting vehicles.

The company argued that it had spent heavily on promoting the name Budget, and that the trademark had been used widely and intensively. But the registry decided that *budget* could not be treated as distinctive in law, as the word had acquired the meaning of "cheap" or "inexpensive", and other traders might legitimately wish to use it in promoting their own car hire services.

Nor does the registry allow words that refer directly to the character of goods. Thus applications to register Vapirub (for vapour rubs) and Crunch (for crunchy chocolate bars) have both been rejected. By contrast, words with only an indirect reference to the characteristics of goods, such as Aphrodisia (for soaps and per-

fumes) have been allowed. So have ambiguous words lacking any direct reference to the goods covered, such as Mothercare, signifying equally 'care by mothers' and 'care for mothers'.

Even if initially distinctive, a valuable name can, if not properly handled, lose its trademark significance and become the generic name for the goods or services being provided. If this occurs, the name no longer functions as a trademark, as it does not distinguish the goods of a particular company. Many former trademarks, including *escalator*, *yo yo*, *trampoline* and *gramophone*, have suffered this fate, commonly referred to as 'genericide'.

Once a trademark has been obtained, great care must therefore be taken to ensure that it does not become used generically. The temptation to use a trademark to describe a particular type of goods is risky. If the trademark is used in speech or writing, it should legally be as an adjective ("I've got a Hoover vacuum cleaner"), not as a noun ("I've got a Hoover").

For the trademark owner, the key is choosing a trademark carefully. A name that is too descriptive or too similar to the product involved may make it difficult to argue that, as a trademark, the name distinguishes a particular product. At the same time, unless the trademark has some connection with the product, it may not catch on and establish the desired reputation. Examples of marks that refer to the product covered include Tagamet for SmithKline Beecham's anti-ulcer drug Cimetidine, Weetabix, Fybogel and Walkman.

As for *taxol*, even though the mark has now been successfully registered, this may not be the end of the matter. As other companies with an interest in cancer treatments will be aware, mechanisms exist for revoking trademarks if it is considered that they do not distinguish the products in question.

But more importantly, the success of Bristol-Myers Squibb in registering the mark suggests that there should be a tightening up of the registration procedure. Perhaps the current controversy will provide the impetus for such a move. □

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1. *Nature* **373**, 370; 1995
2. *Nature* **374**, 208; 1995

This is one of an occasional series of articles on intellectual property and the law.

Nature's sister journals in review

Highlights this month include a strategy to contain arterial damage, a new gene predisposing to obesity, insight into the role of calcium in the blood clotting cascade and the 'reading-head' activity of DNA-binding proteins.

Obesity and insulin processing in mice

RECENT studies on the genetic causes of obesity in mice have produced some exciting results that are beginning to unravel the complex processes controlling body weight and energy output in humans.

On page 135 of the June issue of *Nature Genetics*, J. K. Naggert and colleagues describe the localization and potential molecular mode of action for the *fat* locus in mice. Peter Keightley, in an accompanying News and Views, gives us an overview of this work and discusses progress towards a better understanding of the genes that predispose an individual to obesity.

The *fat* locus in mice tells a somewhat different story from that presented for the *ob* gene nearly six months ago. Mice homozygous for a *fat* mutation grow obese later than *ob/ob* mice. The *ob/ob* mice can be distinguished from their littermates at one month of age; the *fat/fat* mice are not significantly obese until 8–12 weeks of age. Furthermore, *fat/fat* mice have chronically high levels of plasma insulin independent of the onset of hyperglycaemia, whereas serum insulin levels of *ob/ob* mutant mice decline with increased blood glucose levels. Surprisingly, when *fat/fat* mice were given exogenous insulin, their serum glucose level fell. This unexpected result is explained by the finding that 70% of the high serum 'insulin' in *fat/fat* mice was proinsulin, a biologically less active form of insulin. This not only explains why endogenous insulin in *fat/fat* mice had little effect on blood glucose levels, but also indicates a role for the *fat* gene in insulin processing. Indeed, *fat* localizes to the vicinity of the gene encoding carboxy-

peptidase E (CPE), which is required for the processing of proinsulins, and may itself turn out to be the *fat* gene.

CPE activity in both pancreatic islets and the pituitary of *fat/fat* mice is 20-fold lower than that in wild-type mice. This is explained by a sequence mutation found in CPE cDNA isolated from total RNA of *fat/fat* mice. The mutation codes for a missense mutation (Ser202Pro) in a highly conserved region of the CPE protein. *In vitro* analysis of rat CPE with the corre-

sponding amino-acid change confirms that this mutant has much lower enzymatic activity than wild type.

How the *fat* gene affects body weight is not clear; it is uncertain how a defect in insulin processing might lead to obesity. The *ob* mutation seems to be a defect in a *trans*-acting peptide directly affecting fat deposition. Naggert and colleagues postulate that the late-onset obesity in *fat/fat* mice is not necessarily caused by the hyperproinsulinaemia, but rather that it may be a result of defects in the processing of other endocrine or neuroendocrine prohormones that are associated with the control of individual nutritional intake and energy output. □

Putting a stop to proliferation

ATHEROSCLEROSIS — the thickening or narrowing of the arteries, found to varying degrees in most of the adult male Western population — can result in angina and, if left untreated, heart attack. When drugs fail, physical remedies can be used to treat the condition. In one of these (the awkwardly named percutaneous transluminal coronary angioplasty, or PTCA) a small balloon is guided to the stenosis and inflated. The obstruction is, in effect, pushed out. Although it has long been recognized that the technique is somewhat brutal (a few per cent of cases require coronary surgery to correct PTCA-induced damage), the procedure has proved quite successful and has been adopted widely. However, a significant drawback to this procedure is the alarming rate of re-stenosis, reported within three to six months of treatment in up to half the cases. Here the patient comes full circle, from the original atherosclerotic event to a subsequent and perhaps more severe atherosclerosis. On page 541 of this month's *Nature Medicine*, C. Indolfi and colleagues look to *ras* proteins to interrupt this cycle of arterial damage and smooth-muscle cell proliferation.

The *ras* proteins are key transducers of extracellular stimuli, forming part of an incompletely understood but seemingly complex chain of signals from plasma membrane to nucleus. *In vitro* experimentation has shown that interfering with *ras* proteins inhibits cell proliferation. Because illegitimate smooth-muscle cell

proliferation is the cause of re-stenosis, Indolfi *et al.* chose to target normal Ras signalling in an animal model of re-stenosis and examine the effect on the artery.

PTCA procedures were performed on the common carotid artery of rats. In some animals, injuries were allowed to develop uninterrupted for 14 days, after which typical stenoses were found. In others, DNA plasmid vectors expressing a *ras* transdominant negative mutant (known to interfere with Ras function) was applied, in a gel matrix, to the artery immediately after injury. On examination 14 days later, there was a significant reduction in stenosis in all treated rats. Closer examination revealed that both the neointima and the ratio of the neointima to the media (the middle layer of the arterial wall) were reduced in the treated rats.

These results clearly demonstrate that a plasmid construct can be used to express a

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mutant *ras* protein and that this can inhibit, presumably by competing with wild-type Ras for cellular targets, smooth-muscle cell proliferation following balloon angioplasty. But it is not yet clear how closely these experiments might reproduce a similar *in vivo* treatment of human re-stenosis. It is likely that the cellular processes in re-stenosis are somewhat different from those seen in the stenosis of the rat model. Treatment of a pre-existing occlusion may involve responses that are not as clearly Ras-dependent. Nor is it clear precisely what part the *ras* proteins

play in the sequence of events leading to stenosis and re-stenosis. *In vitro* experiments have only suggested a general role in cell growth and proliferation. Further work may indicate that a range of growth factors will represent more specific targets for disrupting the cellular mechanisms involved in re-stenosis. On the other hand, the work described by Indolfi and co-workers suggests that a similar antiproliferative treatment might be useful in treating other proliferative disorders such as the initial atherosclerosis that so often leads to re-stenosis. □

Calcium and coagulation

MANY of the enzymes involved in blood clotting must be attached to cell membranes in order to function. This adhesion is achieved by a region of the protein known as the Gla domain and occurs only in the presence of calcium. On page 504 of this month's *Nature Structural Biology*, Johan Stenflo and colleagues show how this calcium-mediated switch works.

Several of the proteins involved in the proteinase cascade that leads to the formation of a blood clot (for example, factors VII, IX and X as well as prothrombin) contain homologous domains of about 50 amino acids. In this domain the amino acid glutamate has been altered by post-translational modification to γ -carboxyglutamic acid (Gla) which is a strong binder of calcium ions. This post-translational modification is achieved in the endoplasmic reticulum by a vitamin K-dependent enzyme. When free in solution, these coagulation factors have a low affinity for their substrates, which often also contain Gla domains (for example, factor X's substrate is prothrombin). Anchoring these proteins to phosphatidylserine-containing membranes increases the local concentration of both enzyme and substrate, greatly increasing the enzyme's activity.

Using nuclear magnetic resonance (NMR) spectroscopy, Stenflo and colleagues have determined the structure of the Gla domain from factor X (in conjunction with its neighbouring epidermal growth factor-like domain) in the absence of calcium. The domain is made from a bundle of three α -helices and a less ordered loop. All the Gla residues, as would be expected for highly charged groups, are fully exposed to the solvent. Unfortunately the structure of the calcium-bound domain could not be determined because of protein aggregation; however, comparison with the highly homologous domain from prothrombin (whose calcium-bound structure had previously been determined by X-ray crys-

tallography) revealed calcium's dramatic effect.

In the presence of calcium, the Gla residues swing into the structure to form an ionic network at the centre of the

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domain with seven calcium ions at its heart. This rearrangement twists the protein backbone, forcing three residues, a phenylala-

nine, a leucine and a valine, which previously formed the hydrophobic core of the domain, out into solution. These three amino acids form a hydrophobic patch on the surface of the domain and it is this that, in order to escape its aqueous surroundings, attaches to cell membranes.

This mechanism of calcium-induced cell adhesion has some parallels in the responses of the proteins annexin and recoverin to calcium. Their structural rearrangements pale in comparison to this example, where the protein domain has been quite literally turned inside out.

Deconstructionism reaches DNA

If an organism's DNA is likened to a data-storage device such as a compact disk, then DNA-binding proteins are the cell's CD player. On page 450 of this month's issue of *Nature Structural Biology*, Dusan Stanojevic and Greg Verdine strip down the transcription activator GCN4 to nothing but its 'reading-head' to understand better the nature of its DNA specificity.

In a large number of DNA-binding proteins the amino acids that directly interact with the DNA are carried on a short stretch of sequence little more than 20 residues long. This sequence frequently forms an α -helix which inserts into the major groove of the DNA. In spite of this, it has proved impossible to study the binding of isolated peptides because they have vanishingly small affinities when compared to the proteins from which they are derived. Despite not being directly involved in DNA binding, the functions of the rest of the molecule, for example providing a scaffold for the 'reading-head' peptide or the formation of dimers and

larger protein oligomers, significantly contribute to the binding energy of the DNA-protein complex.

The protein GCN4 is dimeric and although individual monomers fail to bind DNA, artificially dimerized proteins will bind even when the DNA sequence used is somewhat different from that bound by the native protein. The binding of one half of the dimer holds the other half in place ready to interact with DNA. Stanojevic and Verdine therefore reasoned that if a 'reading-head' peptide were attached to the DNA by a flexible linker, its sequence-specific binding would be allowed to take place in a similar way.

This attachment was achieved by including in the DNA a modified adenine base containing an -SH group which was linked by a disulphide bond to a cysteine residue at the carboxy terminus of the test peptides. Specific binding of the peptide could be detected by a DNase I protection assay or by assaying the stability of the assemblies to reduction. DNA-peptide complexes were placed in conditions that reduced the disulphide linker; if the peptide was only attached by this linker it would quickly diffuse away; however, if the peptide remained bound to the DNA by specific interactions, even transiently, there was a strong chance of the linker reforming.

As predicted, a 'reading-head' peptide derived from wild-type GCN4 bound tightly to an oligonucleotide containing the GCN4 recognition unit, whereas alterations of either the DNA or peptide reduced this interaction, and thus the details of this process could be dissected. Of more import, however, is the potential power of the technique itself. The specifics of DNA recognition can now be studied unencumbered by the complications imposed by protein chemistry using the resistance to disulphide reduction as a simple, sensitive and quantitative assay. Furthermore, the peptides used are small enough that the emerging science of combinatorial chemistry can be used in an attempt to discover 'reading-heads' for any desired DNA sequence. Such peptides could be used to produce DNA-binding proteins of defined specificity to act as powerful tools in medical and molecular biology. □

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